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SEPARATION DYNAMICS AND PHYSICOCHEMICAL ATTRIBUTES OF β-LACTOGLOBULIN AS INFLUENCED BY STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF THE PROTEIN

BY

ANNA MACLEOD ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

IN

FOOD SCIENCE

DEPARTMENT OF FOOD SCIENCE & NUTRITION

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THE UNIVERSITY OF ALBERTA

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ABSTRACT

The objectives of this study included: 1) evaluation of the bioaffinity subunit exchange affinity chromatography (BSEAC) separation dynamics of β-lactoglobulin in pure and complex solutions and 2) examination of structural/functional aspects of the protein in terms of physicochemical properties such as binding of hydrophobic molecules (vitamin A).

Separation of β-lactoglobulin variant A using the tendencies of the protein to self-associate under given environmental conditions was investigated with respect to kinetic parameters of the association reaction i.e. dissociation constant (Kd) and binding ability of the column (Bt). It was determined using a direct plotting method which deals with the data in observation space that more than one Kd intersection point existed. The Kd values were 120 μMol and 104 μMol at pH 4.6 and a temperature of 4°C. Hydrophobic interaction chromatography was utilized to investigate the occurrence of the multiple Kd values and it was determined that both values were valid and due to higher order aggregation.

The effects of temperature and protein concentration on the extraction of β-lactoglobulin variants A and B from whey solutions were examined. It was observed that the separation was very concentration dependent, however, there were no obvious temperature effects. Due to the potential of microbial growth at room temperature, further experiments were conducted at 4°C.

The effect of pH on β-lactoglobulin isolation from whey was also examined. It was determined through hydrophobic interaction chromatography that aggregate size was pH dependent. Particle size distribution profiles were used to examine the effect of pH and temperature on the degree of association/dissociation i.e. aggregate size. Although the highest separation efficiency (78.28%) was achieved at pH 4.6 (4°C), the largest aggregates were noted at pH 5.2. It was therefore proposed that the extraction could be enhanced if the BSEAC trials were: 1) run at pH 5.2 and 2) the column bead size be adjusted to allow passage of larger molecules.

Studies were undertaken to evaluate kinetic parameters such as apparent dissociation constant (Kd) and protein/ligand molar binding ratio (n) of charcoal treated (CT)β-lactoglobulin variants A and B and retinoic acid. β-lactoglobulin variant A was also examined in terms of its ability to withstand peptic hydrolysis. It was concluded that 1) the binding ratio of both genetic variants was 1:1; and 2) CT β-lactoglobulin was resistant to peptic hydrolysis. The bound retinoic acid /native and defatted β-lactoglobulin as well as proteins that were not associated with retinoic acid were assessed in terms of their ability to withstand tryptic hydrolysis at pH 8.1 at 37°C. The retinoic acid bound proteins were much more resistant to trypsin than both the native and charcoal treated (CT) proteins (i.e. digestion of 18% and 92%, respectively.

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I regret that my mother Mrs. Vina Legault will not see me graduate but if she were here, she would have taken me shopping.

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CHAPTER 1

Introduction

Milk, from a chemical point of view, is a complex composed of hundreds of compounds dispersed and/or solubilized within an aqueous phase. Nutritionally, milk contains members from three major food components: protein (~3.2%), fat (~3.7%), and carbohydrate (~4.6%) (1). The ash fraction (~0.72%) is made up of micronutrients such as calcium, magnesium, chloride and phosphorous (1). Also available through the consumption of dairy products are fat soluble vitamins such as A and D.

There is a current trend to examine food products not just in terms of their traditional applications and nutritional profile but also for potential use of the individual components in novel food products and pharmaceuticals. The term nutraceutical, which was proposed by the Foundation for Innovation in Medicine (Cranford, N.J.), summarizes the concept of using nutritional or food components

for the prevention and treatment of disease.

Milk components, because of their availability, composition and biological activity, are an excellent medium for exploration, and in the future, perhaps an excellent resource for "white mining".

1.1. PROTEIN DISTRIBUTION IN MILK

The protein fraction in milk is responsible for many of the functional characteristics utilized in the manufacture of dairy products such as cheese, yoghurt, sour cream and buttermilk. It is through manipulation of environmental and physical parameters such as pH, temperature and mechanical stress that protein performance can be altered.

Milk proteins can be divided into two major categories: 1) casein (~85.0% of milk protein) and 2) whey (~15.0% of total milk protein) (1). Casein fractions can be described as heat stable, pH sensitive, random coil structures (2). Whey proteins, on the other hand, are heat sensitive, pH stable, containing secondary structure such as α-helix and β-pleated sheets (2). Whey is the solution that remains once the casein fraction has been precipitated through either enzymatic means or by adjustment of pH. Liquid whey and consequently whey proteins are a by-product of cheese manufacture making whey an economical source of "functional" proteins.

1.1.1 WHEY PROTEINS

The whey fraction is made up of different categories of proteins: 1) β-lactoglobulin (~66%); 2) α-lactalbumin (~22%); 3) immunoglobulins (~10%); 4) bovine serum albumin (~6%); 5) lactoferrin and 6) enzymes (1). There are subcategories in each of the above fractions. For example, there are many different enzymes present in milk i.e. lipases, proteases, alkaline phosphatase, catalase, lactoperoxidase, etc.

The albumin protein is the soluble portion of the enzyme lactose synthetase. The immunoglobulins, IgM, IgA and IgG (various) are partially responsible for the natural antimicrobial properties both within the milk itself and in the consuming mammal. The enzymes are biochemical catalysts and serve a variety of functions such as the break down of the primary components i.e. fat and protein.

The whey protein fraction was typically dealt with as a whole, but the individual fractions and their relative functionalities are currently receiving more attention. While the other whey proteins have both physiological and functional characteristics, β-lactoglobulin, the most abundant protein in whey, was the focus in this body of work.

1.2 β-LACTOGLOBULIN

β-lactoglobulin may be present in 4 different genetic forms - variants A, B, C and D with A and B being most common in North America, C exclusive to the

Jersey breed and D having specific European origin (1). α-lactalbumin is found predominantly as variant B with the only exception being the Zebus breed where variant A is predominant. The expression of β-lactoglobulin variants is controlled by genes which are autosomal alleles without dominance (3). β-lactoglobulin variant monomers have an approximate molecular weight of 18,000, an isoelectric point around pH 5.3 and a tendency to self-associate. The degree of self-association varies between the different genetic variants with variant A having the strongest tendency to agglomerate.

1.2.1 STRUCTURE

Papiz et al. (4) examined the structural conformation of β-lactoglobulin at pH 7.6 and noted that it had an unusual protein fold composed of two slabs of antiparallel β-pleated sheet. The pocket formed by the β-sheets was compared with the fold or pocket of plasma retinol-binding protein (4,5). While the physiological function of β-lactoglobulin has not been fully elucidated, structural interpretations suggest that this protein is able to bind hydrophobic molecules. Since β-lactoglobulin is stable under acidic conditions, it has been suggested that it may serve to transport molecules through the low pH's encountered in the stomach (4,6).

Heating, dielectric constant, pH and combinations thereof have been utilized to study the response of β-lactoglobulin to varied physical and chemical conditions (6,7,8,9,10,11,12). Heat treatment of β-lactoglobulin causes denaturation of the

native structure with the degree of denaturation being time/temperature dependent (6,7,8). Denaturation causes changes in the secondary and tertiary conformation of the protein and hence alters it's ability to self-associate and bind macromolecules. Characteristic properties of β-lactoglobulin such as resistance to enzymatic digestion and the formation of higher order structures have all been examined (6,7,8).

Structural transformation of β-lactoglobulin observed by alteration of dielectric constant has also been studied (9,10). Primarily, the effect of dielectric constant on the β-barrel, the tridimensional structural pattern common to many hydrophobic molecule transporters, was the focal point of the investigations (9,10). It was concluded that temporary changes in secondary structure caused by lowering the dielectric constant ($\acute{\varepsilon}$ = 50) altered the ability of β-lactoglobulin to bind retinol, a hydrophobic, fat soluble vitamin (9,10). The binding affinity could be restored once the dielectric constant was raised back to $\acute{\varepsilon}$ = 70 (10). Heating causes permanent changes to the secondary structure thereby permanently altering hydrophobic macromolecule binding behaviour while variations in the dielectric constant only cause transient modifications to the structure of the β-barrel (9,10). It is through structural manipulation of β-lactoglobulin that the importance of some of the conformational attributes such as the β-barrel structure have been elucidated.

pH has been shown to have a significant effect on the conformation of β-lactoglobulin. The monomeric form of β-lactoglobulin is predominant at pH<3.7 and

at pH >5.4. Between pH 3.7 and 5.4 the protein undergoes temperature dependent tetramerization and above pH 7.0 the protein tends to slowly denature (11,12,13,14,15). The functional characteristics of β-lactoglobulin are dependent on the structural and conformational attributes of the protein. Temperature, dielectric constant and pH are all tools which can be utilized to modify the structure of β-lactoglobulin thereby altering its behaviour.

1.2.2 FUNCTIONALITY

1.2.2.1 β-LACTOGLOBULIN AS A FOOD INGREDIENT

The physical state of β-lactoglobulin has a significant influence over the application of the protein as a food ingredient. Undenatured, native, β-lactoglobulin has good emulsification capacity and whipping/foaming properties while the denatured form is associated with gelation (16). The manufacturing procedures for traditional products such as yoghurt rely on changes in protein functionality through temperature and pH manipulation (16,17,18).

Lipophilization, alcohol modification, reductive alkylation, chemical or enzymatic attachment of aromatic amino acids and phosphorylation are methods which have been investigated to improve the functional characteristics (emulsifying and/or foaming) of β-lactoglobulin (16,18,19,20).

1.2.2.2 PHYSIOLOGICAL ACTIVITY OF β-LACTOGLOBULIN

Although the physiological role of β-lactoglobulin has not been completly elucidated, studies have linked the structure/function of the protein with its physicochemical properties. At the root of most of the proposed biological activity is the ability of β-lactoglobulin to bind hydrophobic molecules. Two different binding sites have been proposed: 1) the interior β-barrel and 2) exterior hydrophobic surface cleft (5). According to Cho et al. (5) the retinol binding site is in the interior cavity.

A few of the physicochemical activities that β-lactoglobulin has been associated with are: 1) binding of retinoids (21, 22); 2) binding of β-carotene (21); 3) binding of fatty acids and triglycerides (22,23,24,25,26) and 4) interaction with pregastric lipase (26). The binding stoichiometry of retinoids to β-lactoglobulin has been proposed to be 1:1 based on molar ratios (21,22). Dufour and Haertle (21) also examined β-carotene binding with β-lactoglobulin and found the ratio to be 1:2 (ligand:protein), however increased binding affinity was reportedly achieved through esterification and alkylation of the protein. As previously mentioned, there is a high degree of homology in the amino acid sequence and conformational structure (β-barrel) between human plasma retinol binding protein and β-lactoglobulin. Puyol et al. (1991) found that palmitic acid competes with retinol for the binding site at concentrations similar to those found in milk (22). Physiological implications aside, there is potential for use of β-lactoglobulin as a retinol carrier in vitamin

preparations, low fat food formulations and cosmetics because when bound, the hydrophobic molecules may be: 1) sheltered from oxidative reactions and 2) water miscible.

The interaction between lipids and β-lactoglobulin has received considerable research attention in the past five years. Much like retinol binding, the lipid interaction has been characterized *in vitro*, however, there is little definitive data regarding the physiological implications of the association (24,25,26). Perez et al. (26) have proposed that one function may involve modulation of pregastric lipase through the removal of fatty acids which naturally inhibit the enzyme. Despite any physicochemical role, increased hydrophobicity of the lipid bound β-lactoglobulin is responsible for some of the functional characteristics of the protein i.e. emulsifying and foaming capabilities (16).

1.3 METHODS OF SEPARATING β-LACTOGLOBULIN

There are many techniques that can be employed to separate β-lactoglobulin from the other whey protein fractions. The selective removal of β-lactoglobulin could serve a dual purpose: 1) decrease the allergenicity of whey as β-lactoglobulin has been implicated with milk protein allergenicity (27) and 2) isolate a protein which has potential as a nutraceutical. Due to the nature of the applications (food and pharmaceuticals), the protein fraction should be separated

using GRAS reagents and procedures.

1.3.1 FUNCTIONAL

Chromatography is an analytical technique used for the separation of molecules within a gas or liquid mobile phase. Chromatography is relatively simple, efficient with a large range of applications. There are a number of chromatographic venues which have been applied to the examination and separation of β-lactoglobulin, however, the most successful method for extraction was biospecific subunit exchange affinity chromatography (BSEAC) (15,28,29,30). Affinity chromatography is an adsorption type chromatography where the molecule to be purified is specifically and reversibly adsorbed by a ligand (complimentary binding substance) which is immobilized on a support matrix. In the case of β-lactoglobulin, the ligand is β-lactoglobulin itself and the procedure, therefore, relies on the natural ability of β-lactoglobulin to self-associate under certain environmental conditions (15, 28).

1.3.2 PHYSICAL

Physical means, such as centrifugation and ultrafiltration, for separating β-

lactoglobulin from whey are not effective because of contamination with α-lactalbumin. The molecular weight of α-lactalbumin is 15,100 Daltons while the molecular weight of β-lactoglobulin is 18,000 Daltons causing co-extraction of the proteins (1).

1.3.3 CHEMICAL

One method that has been widely used for the separation of proteins is salting in/ salting out. Salts are used to change the ionic environment thus changing protein solubilities. In general, three steps are required: 1) changing ionic strength; 2) centrifugation and 3) removal of salts (diafiltration, dialysis, chromatography, etc.). This methodology has been used in the study and separation of β-lactoglobulin (31,32).

To effectively and efficiently separate β-lactoglobulin from whey, a combination of physical and chemical steps are typically employed. BSEAC, on the other hand, relies on the intrinsic protein functionality thereby streamlining the separation while avoiding the use of solvents and other undesirable reagents.

1.4 RESEARCH OBJECTIVES

Cheese whey is a mixture of lactose, protein, fat and minerals, therefore, the

specific isolation of a protein such as β-lactoglobulin is not without its' challenges. The potential application of the separated fraction must also be considered when designing the isolation protocol. The criteria that was outlined for the β-lactoglobulin separation were as follows: 1) the protein must remain in the native state because the conformation of the protein is critical in terms of retaining the ability to bind micronutrients such as retinol (21,22); 2) the use of organic solvents and other non-food grade chemicals must be avoided because of the potential applications i.e. nutraceuticals and functional foods and 3) there must be potential commercialization of the process.

Biospecific subunit exchange affinity chromatography (BSEAC) was chosen as the extraction method to be investigated because the separation criteria outlined above, could be met using this methodology. BSEAC relies on the natural ability of β-lactoglobulin to self-associate under given pH and temperature conditions.

The overall objectives of the research were: 1) define the kinetics of a BSEAC system with respect to dissociation constants and binding/separation potentials using a pure variant; 2) investigate the separation of β-lactoglobulin from whey under varied environmental circumstances; 3) examine the resultant separation efficiencies; 4) define parameters for optimization and 5) examine the nutraceutical and physiological potential (e.g. retinol binding affinity) of the β-lactoglobulin. In short, separation methodology and potential application of the isolated protein were the focal points of the investigation.

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CHAPTER 2

Separation of β-Lactoglobulin A By Biospecific Subunit Exchange Affinity Chromatography[1]

2.1. INTRODUCTION

Cow's milk, like all mammalian milk, consists chiefly of water, fat, carbohydrate, protein and minerals, however, the relative proportions of each of the constituents vary as much as the species themselves(1). From a nutritional standpoint, milk is ideally suited for the producing species but from a manufacturing and human feeding perspective, manipulation of the constituents may be advantageous.

Focusing on the protein fraction of cow's milk, it is the composition and the relative proportions of the various components that give bovine milk its' unique physiochemical, biochemical and nutritional properties (2). The physiochemical

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1 A version of this chapter has been accepted for publication. MacLeod & Ozimek 1995. Milchwissenschaft. 00 000-000.

and biochemical properties may be enhanced through physical and/or chemical modification (3,4,5). The nutritional or in some cases the antinutritional factors, may also be altered through structural modification or by changing the composition via separation technology (6,7,8,9).

Milk proteins can be divided into two major categories: 1) casein and 2) whey. The largest opportunity for the exploitation of dairy proteins exists in the whey protein fraction because during standard manufacturing of cheeses such as cottage and cheddar, 90% of the initial milk volume ends up as a by-product known as whey. In general, whey proteins are sensitive to denaturation above 60°C and their sensitivity is effected by ionic strength and pH. Relying on the physiochemical properties which are predictors of functionality, it is possible to separate the individual whey protein fractions. For example, β-lactoglobulin, which represents approximately half of the whey proteins tends to tetramerize between pH 3.7 and 5.2; the degree of tetramer formation is also influenced by ionic strength and temperature (10). Chiancone and Gattoni (11) proposed sub-unit exchange chromatography as a means of selectively isolating the β-lactoglobulin from whey by utilizing the association-dissociation phenomena of this protein fraction. According to Chiancone and Gattoni (11) the protein was recovered in native form.

The primary objective of the present work was to evaluate the separation of β-lactoglobulin A by biospecific subunit exchange affinity chromatography and to

establish the kinetics of the sub-unit exchange.

2.2. MATERIALS AND METHODS

2.2.1. CHEMICALS

CNBr-activated Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden); β-lactoglobulin genetic variant A was a crystallized product from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2.2.2. AFFINITY CHROMATOGRAPHY

The apparatus that was used consisted of the following Pharmacia FPLC components: Single path UV-1 monitor and control unit (λ=280 nm), P-500 pump, GP-250 gradient programmer, Rec-481 single channel recorder, Frac-100 fraction collector and a Cyanogen Bromide Sepharose 4B matrix for β-lactoglobulin binding.

2.2.2.1. COLUMN PREPARATION

The methodology of Tercero and Diaz-Maurino (12) for coupling to S-4B matrix was utilized with some modifications. Freeze dried S-CNBr-4B was hydrated directly in the column casing; 0.403 g of the matrix was washed by pumping first 0.1 M HCl for 60 minutes at 2.5 ml/min and 20°C to wash out additives and

preservatives. Coupling of the β-lactoglobulin A to the CNBr was accomplished by dissolving the protein in coupling buffer (0.1 M $NaHCO_3$ containing 1.0 M NaCl at pH 7.0) and the solution was recirculated at a flow rate of 0.3 ml/min for 17 h at 4°C. The excess ligand was washed away with coupling buffer followed by neutralization of the remaining active groups using 0.1 M ethanolamine, pH 9.5 for 24 h at 4°C.

2.2.2.2. SAMPLE PREPARATION

The β-lactoglobulin A was dissolved in associating buffer (0.1 M Na acetate at pH 4.6); the concentration of protein was varied between 0 and 1600 µmols. The samples were centrifuged at 10,000 X g for 25 min at room temperature to remove any particulate matter. Protein concentration and purity were verified prior to application to the affinity chromatography system using the HPLC methodology outlined below. The samples were applied to the column using an 11.4 ml loop.

2.2.2.3. OPERATING CONDITIONS

The samples were applied to the Pharmacia FPLC apparatus described above at a constant flow rate of 0.3 ml/min at 4°C; the protein concentration was monitored using U.V. absorbance at 280 nm and integral boundary information was determined e.g. leading centroid volume (equilibrium) and plateau (column saturation)(2). The dissociating buffer (0.1 M NaCl-HCl, pH 2.0) was applied to the column and the absorbance of the peak of eluted protein was measured.

2.2.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The liquid chromatography system used consisted of the following Shimadzu components: SIL-6A auto injector unit, C-R6A Chromatopac integrator, SCL-6A system controller, LC-6A binary pumping system, SPD 10A dual channel detector (Fisher Scientific, Edmonton, Alberta, Canada).

2.2.3.1. β-LACTOGLOBULIN ANALYSIS

A standard curve for β-lactoglobulin A was prepared using the integrated peak areas of solutions that ranged in concentration between 0 and 11.2 mg/ml; all samples were run in triplicate. Best fit standard curves were prepared by linear regression of peak area using Cricket Graph (Cricket Software, Malvern, PA, USA). The column used was a Mono Q (Pharmacia, Uppsala, Sweden) and the mobile phase was 20 mM Tris-HCl, pH 7.0 that was filtered through a 0.22 µm nylon membrane filter (Micron Separations Inc., Westboro MA, USA) and degassed under vacuum. The elution buffer used was 20 mM Tris-HCl, 1 M NaCl, pH 7.0 which had been prepared using the above procedures. The elution gradient used was a step-wise program: 1) from 0 to 4 minutes, 100% buffer A; 2) 5 to 24 minutes, a linear gradient from 0% to 35% buffer B; 3) 25 to 28 minutes, 35% buffer B; 4) 29 to 32 minutes, a linear gradient from 35% to 100% buffer B; 5) 32 to 45 minutes, 100% buffer B and 6) 46 to 50 minutes, 0% buffer B (Alberta Dairy Association Research

Unit laboratory procedure). The flow rate was 1 ml/min with a total running time of 50 minutes. The initial concentrations of all samples applied to the affinity apparatus were verified using the above standard curves and operating conditions.

2.2.4. KINETICS

The centroid volume coordinate of the leading edge (mm) was plotted against the total volume (elution plus void) to determine if there was a linear relationship between β-lactoglobulin concentration and elution profile (13,14,16). The regression coefficient was calculated using Sigma Plot. The two main kinetic attributes of the system focused upon were: 1) the dissociation constant (Kd) of the β-lactoglobulin A bound to the immobilized β-lactoglobulin A and 2) the total amount of β-lactoglobulin retaining binding ability in the column (Bt) (15). The Kd and Bt were determined using two different graphical methods: 1) double reciprocal plot and 2) direct linear plot (16) using Sigma Plot (16).

2.2.5. DETERMINATION OF HIGHER ORDER AGGREGATION

The HPLC apparatus discussed above was used with the exception of the column; a Pharmacia (Uppsala, Sweden) Phenyl-Superose 5/5 HR was utilized.

The hydrophobic interaction chromatographic separation was performed as described by Grinberg et al. (18) with the two following modifications: 1) the choice of hydrophobic column i.e. phenyl versus a methyl polyether phase and 2) the mobile and elution buffers were modified to accommodate the increased hydrophobicity of the Phenyl-Superose. The mobile phase consisted of 0.05 M Tris-HCl and 1.27 M ammonium sulfate and the elution buffer was made up of 0.05 M Tris-HCl in 35 % acetonitrile. The samples (10 mg/ml of β-lactoglobulin A) and solvents were prepared as per the procedures outlined in the HPLC section above. The elution profile was monitored using a wavelength of 280 nm, a flow rate of 0.5 ml/min with a total run time of 40 minutes.

2.3. RESULTS AND DISCUSSION

Bioaffinity subunit exchange chromatography exploits two characteristic properties of subunit interactions in oligomeric proteins: 1) the specificity of recognition and 2) the nature of the stabilizing forces (hydrogen bonds, hydrophobic and electrostatic interactions). Due to the stabilizing forces, the reaction can be manipulated using: 1) pH; 2) temperature; 3) ionic strength and 4) dielectric constant. The application of affinity chromatography for separation of β-lactoglobulin A depends upon the biospecific interaction between the β-lactoglobulin A in solution and the β-lactoglobulin immobilized on the matrix. Therefore, conditions for separation were chosen which would maximize the self

association reaction (17).

According to Brumbaugh and Ackers (13), associating systems are characterized by sharp leading and "diffuse" trailing boundaries. The chromatograms obtained from the β-lactoglobulin A bioaffinity separation (chromatograms not shown) exhibited the aforementioned relationship. The variation in elution volume with substrate concentration is the foundation for frontal affinity chromatography as it is the change in total volume required for the column system to reach equilibrium. The binding isotherms of the β-lactoglobulin A were compared by plotting total volume (elution plus void) against distance required to establish the frontal centroid position for each individual concentration. The β-lactoglobulin bioaffinity separations had a regression coefficient of 1.00 (Figure 2.1) indicating that this system was ideally suited for frontal analysis.

Two graphical methods were used to determine the kinetic constants, Bt and Kd for the β-lactoglobulin A bioaffinity system: 1) a method which dealt with the data in observation space (double reciprocal plot, Figure 2.2) (19) and 2) a technique which relies on the data in parameter space (direct linear plot, Figure 2.3) (20). The total amount of reacting ligand or Bt was very similar when comparing the double reciprocal plot versus the direct linear plot (0.55 μMol/ml versus 0.56 μMol/ml, respectively). The dissociation constant (Kd) for the reaction was not consistent between methods; a value of 154.8 μMol was determined using the double reciprocal technique while two values were observed on the direct linear plot (120

μMol and 104 μMol/ml). Results presented in the form of a double reciprocal plot do not in general appear to be precise because there is mathematical manipulation of the data (16). The Kd and Bt values determined by this technique were a composite of all of the individual binding isotherms as expressed by one single regression line. The direct linear plot, on the other hand, requires no mathematical calculations and deals with individual concentrations versus elution volumes separately. Generally, it is more difficult to measure the reaction velocities at the extreme values, however, when either: 1) an average value for all of the individual observations or 2) a regression line is utilized, it is the extreme observations that have the largest influence on the determined result. With the direct linear approach, it is the median Kd value in the observation space which is utilized, therefore the effect of aberrant observations is diminished (16).

Using the direct linear plot, two distinct Kd intersection points were observed. This observation was thought to be due to higher order interaction of the β-lactoglobulin A. β-lactoglobulin A undergoes reversible higher order polymerization (dimers, tetramers, octamers) which is maximized between pH 4.40 and 4.65 under cold conditions and this phenomenon is concentration dependent (6). To confirm that the two distinct Kd values were the result of higher order aggregation, hydrophobic interaction chromatography (HIC) was carried out using pH and temperature values which reflected the bioaffinity system (i.e. an association pH of 4.6, a dissociation pH of 2.0 and a temperature of 3°C). Two peaks were observed

with retention times of 24.45 and 27.16 minutes when the β-lactoglobulin A was examined on the hydrophobic column at pH 4.6 at 3°C (Figure 2.4). On the other hand, at pH 2.0, the pH which favours monomer formation, the β-lactoglobulin eluted as a single peak at 31.80 minutes (Figure 2.5). Using the nature of the β-lactoglobulin, its characteristic behaviour as environmental factors are modified and the HIC profiles, it was concluded that both Kd values were valid.

If the double reciprocal plot was used exclusively, the calculated Kd would have been within the correct range, however, the occurrence of the two separate Kd values and hence the overall dynamics of the interaction would have been overlooked.

2.4. CONCLUSIONS

In conclusion, immobilized β-lactoglobulin A preserved biospecific interaction with the soluble form of the ligand and the binding was reversible under defined disassociation conditions. The HIC confirmed that under the conditions of the experiment, the soluble subunits of β-lactoglobulin A interacted with themselves as well as the immobilized protein.

During the current study, a pure system consisting of β-lactoglobulin A was utilized, however, the raw material would generally be a complex solution such as milk or whey with varied components such as: water, other proteins, fat, carbohydrate and ash. Studies to characterize the biospecific subunit exchange

affinity chromatography of β-lactoglobulin in a complex matrix are in progress. The information generated in this study could be the basis for defining not only the dynamics of more complex bioaffinity reactions but also to optimize the separation efficiency and determine the feasibility of industrial application.

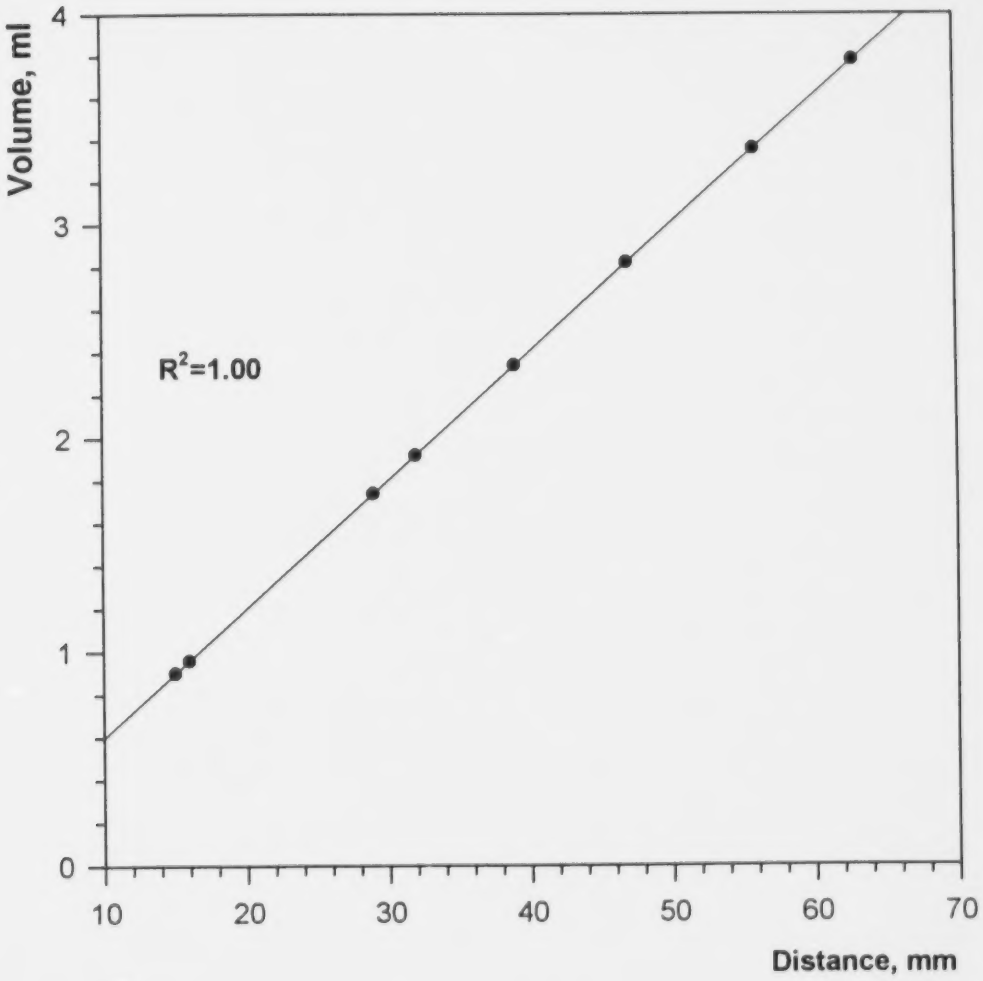

Figure 2.1 Graphical representation of the correlation between centroid position of the chromatographic leading edge (mm) and total volume (ml) for a pure β-lactoglobulin A bioaffinity separation

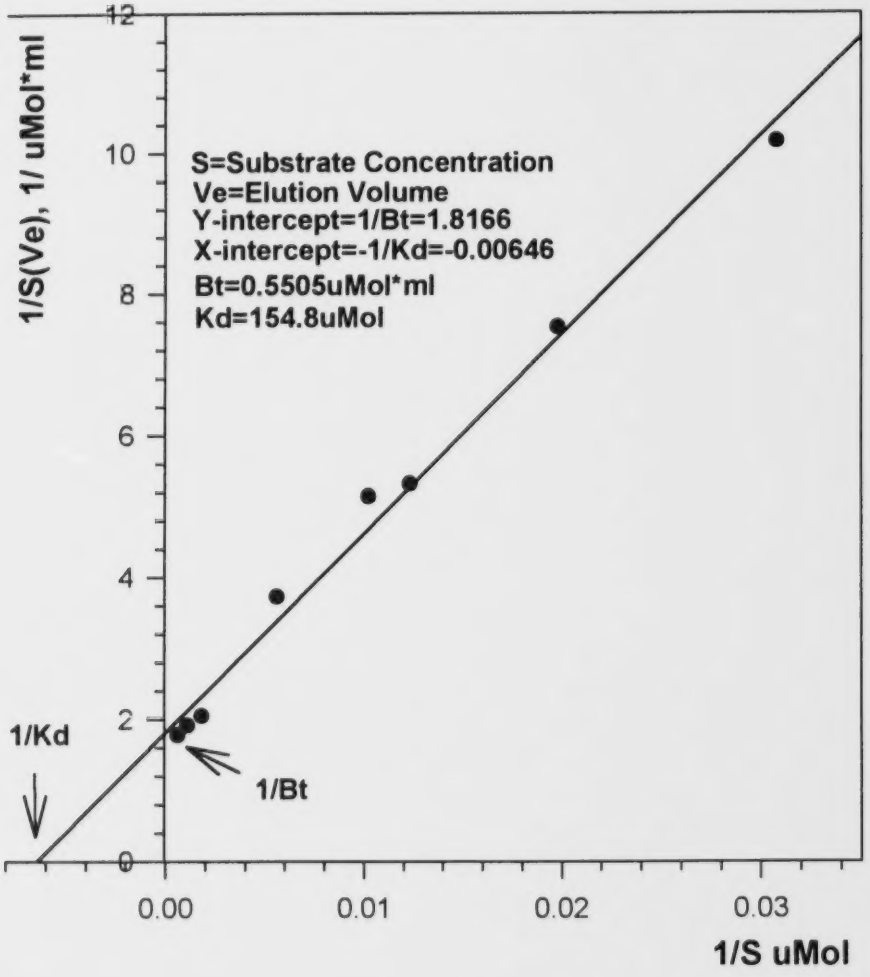

Figure 2.2 Double reciprocal plot of elution volume and substrate concentration for bioaffinity chromatographic data for β-lactoglobulin variant A

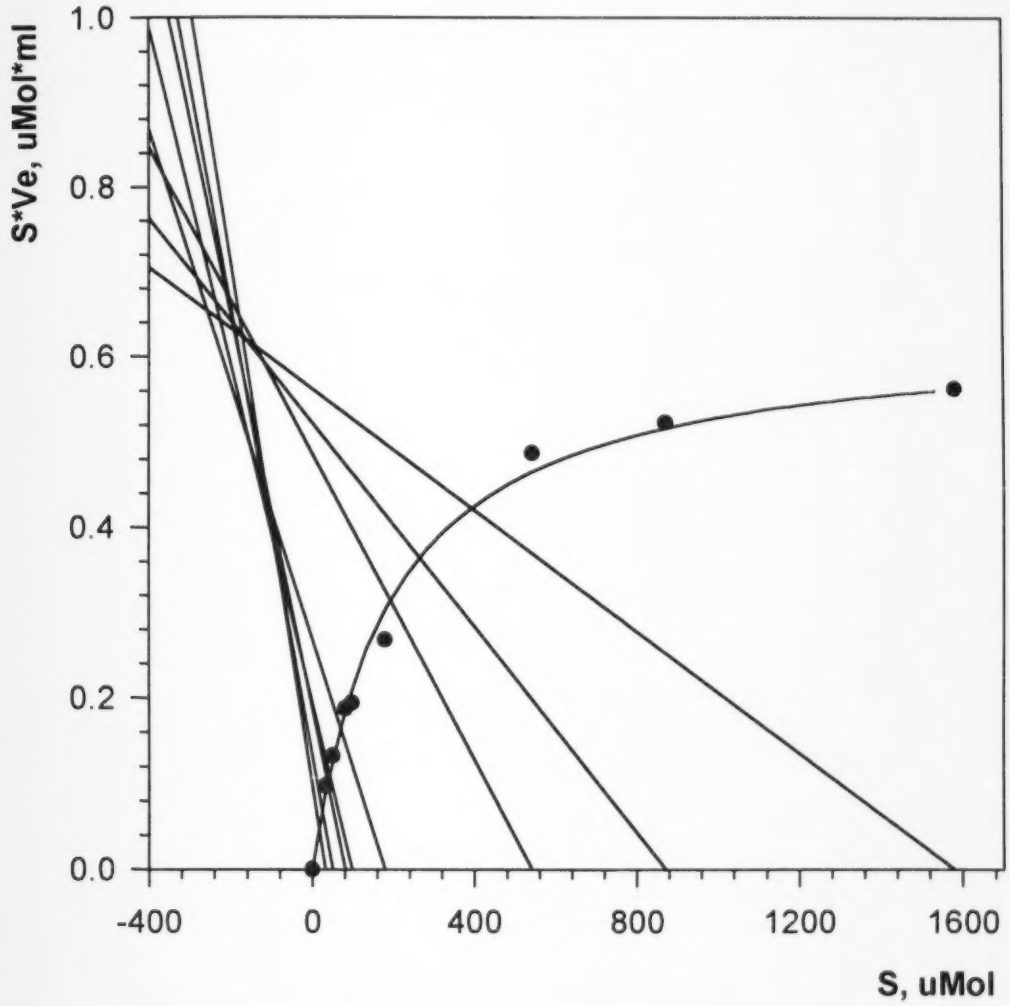

Figure 2.3 Graphical determination (direct linear plot) of Kd and Bt values for β-lactoglobulin variant A as measured by bioaffinity chromatography

CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 1008

PKNO	TIME	AREA	MK	IDNO
1	4.858	89231	V	
		712hi		
2	19.633	2630921	V	
		4680hi		
3	24.45	7590731	V	
		108464hi		
4	27.158	2013481	V	
		15848hi		
	TOTAL	12324364		

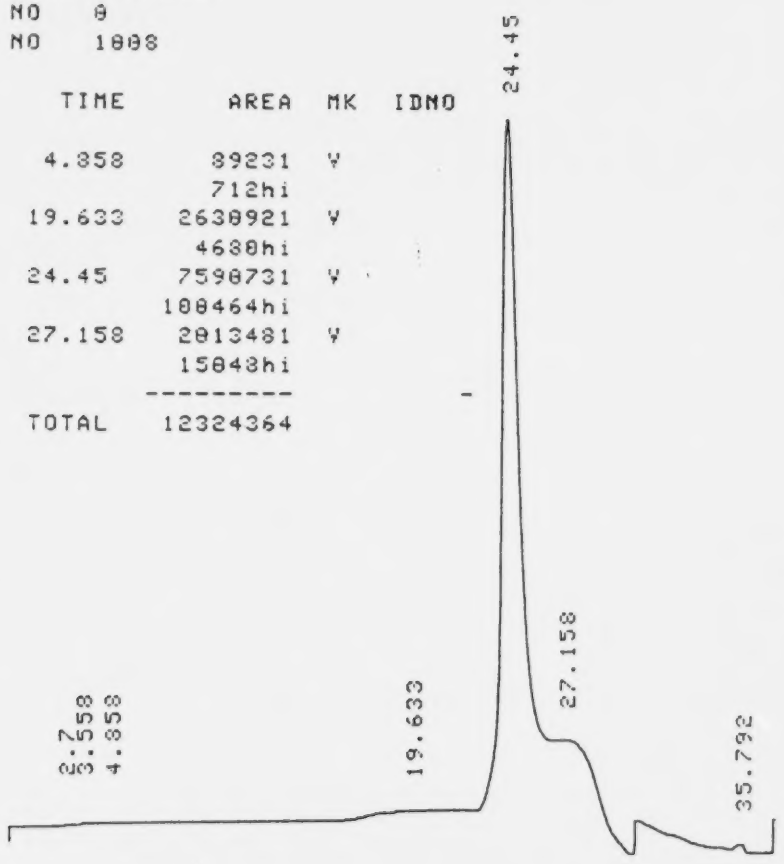

Figure 2.4 HIC elution profile at 3°C of 10 mg/ml β-lactoglobulin A dissolved in mobile phase A, 0.05M Tris-HCl and 1.27M $(NH_4)_2SO_4$ at pH 4.6 using a linear gradient of 100% A to 100% B for 17 minutes. B was the elution buffer consisting of 0.05M Tris-HCl in 35% acetonitrile

CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 1015

FILE 9
METHOD 2041

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	31.382	5565966 37618hi	E		100	
	TOTAL	5565966			100	

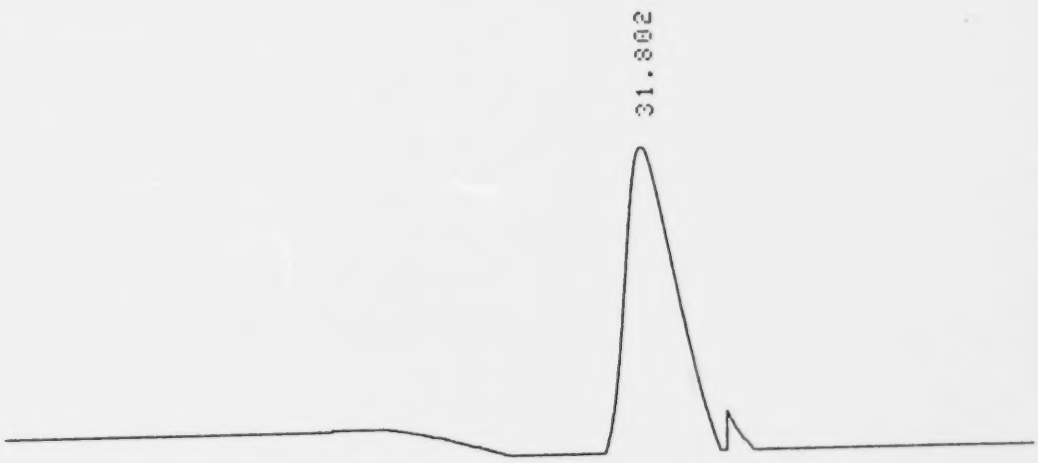

Figure 2.5 HIC elution profile of 10 mg/ml of β-lactoglobulin A using mobile phases A and B that were described in Figure 2.4 with the exception of pH (pH 2.0)

2.5. REFERENCES

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CHAPTER 3

Separation of β-lactoglobulin variants A and B from Cheese Whey By Subunit Exchange Bioaffinity Chromatography[1]

3.1. INTRODUCTION

β-lactoglobulin has been linked to a number of physiological activities such as retinol binding (1,2,3,4), binding with β-carotene (4) and allergenicity (5,6,7). The selective removal of β-lactoglobulin from whey could serve a dual purpose: 1) to provide a substrate that could be used for the manufacture of "functional" foods (eg. allergy reduced infant formula) and 2) to isolate a protein which has potential nutraceutical applications (eg. retinol carrier in food systems and/or in vitamin preparations).

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1 Portions of this chapter have been accepted for publication, MACLOED, FEDIO & OZIMEK , 1995, Milchwissenschaft **00** 000-000.

The focus of this study was to selectively isolate β-lactoglobulin from whey using subunit exchange bioaffinity chromatography. Since whey is a complex matrix consisting of carbohydrates, minerals, fats and proteins, the conditions required for extraction as well as separation efficiencies were examined.

3.2. MATERIALS & METHODS

3.2.1. CHEMICALS

CNBR-activated Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden); β-lactoglobulin genetic variant A and B were crystallized products from Sigma Chemical Co. (St. Louis, MO, U.S.A.); the cheese whey was a hygroscopic powder obtained from Alpha Milk Company (Red Deer, AB, Canada).

3.2.2. AFFINITY CHROMATOGRAPHY SYSTEM

The apparatus that was used consisted of the following Pharmacia FPLC components: Single path UV-1 monitor and control unit (λ=280 nm), P-500 pump, GP-250 gradient programmer, Rec-481 single channel recorder, Frac-100 fraction collector and a Cyanogen Bromide Sepharose 4B matrix for β-lactoglobulin binding

3.2.2.1. COLUMN PREPARATION.

The methodology of Tercero and Diaz-Maurino (8) for coupling to S-4B matrix was utilized with some modifications. Freeze dried S-CNBr-4B was hydrated directly in the column casing; the matrix was washed by pumping first 0.1 M HCl for 60 minutes at 2.5 ml/min and 22°C to wash out additives and preservatives. Coupling of the β-lactoglobulin B to the CNBr was accomplished by dissolving 0.111 g of the protein in 20 ml of coupling buffer (0.1 M $NaHCO_3$ containing 1.0 M NaCl at pH 7.0) and the solution was applied to the column and recirculated at a flow rate of 0.5 ml/min for 2 h at 22°C. The excess ligand was washed away with coupling buffer followed by neutralization of the remaining active groups using 0.1 M ethanolamine, pH 9.5 for 4 h at 22°C.

3.2.2.2. SAMPLE PREPARATION.

Whey powder was dissolved in water and centrifuged at 10,000 X g for 25 minutes at room temperature to remove any particulate matter. The supernatant was adjusted with sodium acetate and HCl to obtain the same molar concentration and pH as the associating buffer (0.1 M Na acetate at pH 4.6); the total amount of whey proteins loaded on to the column varied and corresponded to total β-lactoglobulin loads in the 2 to 90 mg range. In one of the experiments the pH of the associating buffer was increased between 3.5 and 5.2 in 0.2 increments; two

additional samples were run at pH 5.6 and 6.6. The salt concentration was kept constant in all of the trials (0.1 M Na acetate). It was necessary to dissolve the whey in water rather than associating buffer directly, as the β-lactoglobulin would aggregate and be lost during centrifugation at the higher inclusion levels. The concentration of β-lactoglobulin applied to the column in the various whey preparations was verified using the HPLC methodology outlined below. The samples were applied to the column using a 3-way valve.

3.2.2.3. OPERATING CONDITIONS.

The samples were applied to the Pharmacia FPLC apparatus described above at a constant flow rate of 0.5 ml/min at either 4° or 22° C and the sample volume for each run was 52.0 ml. The dissociating buffer (0.1 M NaCl-HCl, pH 2.0) was applied to the column and the peak absorbance was used to determine the total amount of the eluted protein. The total concentration of β-lactoglobulin variants A and B in the peak were determined using the methodology outlined in this paper.

3.2.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The liquid chromatography system used consisted of the following Shimadzu components: SIL-6A auto injector unit, C-R6A Chromatopac integrator, SCL-6A

system controller, LC-6A binary pumping system, SPD 10A dual channel detector (Fisher Scientific, Edmonton, Alberta, Canada).

3.2.3.1 β-LACTOGLOBULIN ANAYSIS

The column used was a Mono Q (Pharmacia, Uppsala, Sweden) and the mobile phase was 20 mM Tris-HCl, pH 7.0 that was filtered through a 0.22 µm nylon membrane filter (Micron Separations Inc., Westboro, MA, USA) and degassed under vacuum. The elution buffer used was 20 mM Tris-HCl, 1 M NaCl, pH 7.0 which had been prepared using the above procedures. The elution gradient was a step-wise program: 1) from 0 to 4 minutes, 100% buffer A; 2) 5-24 minutes, a linear gradient from 0% to 35% buffer B; 3) 25-28 minutes, 35% buffer B; 4) 29-32 minutes, a linear gradient from 35% to 100% buffer B and 6) 46 to 50 minutes, 0% buffer B (Alberta Dairy Association Research Unit laboratory procedure). The flow rate was 1 ml/min with a total running time of 50 minutes. Protein concentration was monitored by measuring the absorbance at 280 nm. A standard curve for β-lactoglobulin was prepared using the integrated peak areas of solutions that ranged in concentration between 0 and 10 mg/ml; all samples were run in triplicate. Best fit standard curves were prepared by linear regression of the peak areas using Cricket Graph. The initial concentrations as well as the protein recovered in the disassociation peaks were verified using the above standard curves and operating conditions.

3.2.4. KINETICS

The two main kinetic attributes of the system focused upon were: 1) the dissociation constant (Kd) of the β-lactoglobulin bound to the immobilized β-lactoglobulin B and 2) the total amount of β-lactoglobulin retaining binding ability in the column (Bt) (9). The Kd and Bt were determined using two different graphical methods: 1) double reciprocal plot and 2) direct linear plot (10) using Sigma Plot .

3.2.5. SEPARATION DYNAMICS

The efficiency of β-lactoglobulin variants A and B extraction from whey was calculated using the following: Separation efficiency (S.E.) = (mg β-lactoglobulin recovered/mg β-lactoglobulin applied) x 100. Experimental trials that varied both the concentration of protein applied to the affinity column and the pH of the associating buffer were plotted in 3D fashion using Stanford Graphics to determine the surface response relationship.

3.3. RESULTS & DISCUSSION

Cheese whey is a mixture of lactose, protein, fat and minerals, therefore, the specific isolation of a protein fraction such as β-lactoglobulin is not without its' challenges. The potential application of the separated fraction must also be

considered when designing the isolation protocol. Criteria for the β-lactoglobulin separation were as follows: 1) the protein must remain in the native state because the conformation of the protein is critical in terms of retaining the ability to bind micronutrients such as retinol (1) and 2) the use of organic solvents and other non-food grade chemicals were avoided because of potential applications for the β-lactoglobulin i.e. nutraceuticals and functional foods. Biospecific subunit exchange affinity chromatography (BSEAC) was chosen as the method of extraction because the separation criteria outlined above, could be met using this methodology. BSEAC relies on the natural ability of β-lactoglobulin to self associate under given pH conditions; the association/disassociation dynamics are also influenced by temperature (11). The isolation of β-lactoglobulin was due to interaction between the covalently bound β-lactoglobulin on the sepharose matrix and the protein in solution (11,12); the perceived complicating factors were the other constituents in the whey.

Frontal affinity chromatography (FAC) was used in other studies (11,12) to assess the dynamics of the separation of β-lactoglobulin when the concentration of total protein was varied.

In previous BSEAC experiments with β-lactoglobulin (12), it was concluded that the direct linear plotting method (13) was more appropriate than the double reciprocal plot (14) because the data is dealt with in observation space requiring no mathematical manipulation. The conclusion was further exemplified in this model

(Figures 1 through 4) as the ligand binding numbers (Bt) were not even in the same order of magnitude while in the pure β-lactoglobulin system the Bt numbers were at least comparable (e.g. 0.55 μMol/ml for the double reciprocal plot versus 0.56 μMol/ml for the direct linear observation) (12). Using the double reciprocal plot (Figures 3.1 and 3.2) the Bt values for room temperature and 4°C were 5.33 μMol and 2.96 μMol, respectively while the direct linear results were 0.664 μMol for room temperature and 0.653 μMol for 4°C. It is difficult to explain the double reciprocal Bt points unless the conclusion is drawn that the mathematical manipulation of the data presents errors which are so large that the affinity system can not be accurately represented using arithmetic transformations. It is assumed that the direct linear method predicts the active bound ligand accurately as the Bt point is the observed saturation of the system.

In addition to defining the kinetics of the BSEAC set-up, the dissociation constant (Kd) is of practical importance because the concentration of protein, in our case β-lactoglobulin specifically, should be above the Kd to achieve the maximum binding velocity. The Kd results for the double reciprocal plots were 0.36 μMol for room temperature and 0.18 μMol for 4°C, Figures 3.1 and 3.2, respectively. The results from the direct linear plots were considerably less conclusive as there were no distinct Kd points, only regions of increased intersection. On both the room temperature and 4°C direct linear graphs, the plots were extended to determine if the intersection points were outside of the plotted values (Figure 3.3 and 3.4). In

both cases, there were no definitive intersections, however, there were 5 regions that had increased cross-section. When the mean Kd values from each area (5 from each graph) were averaged, the Kd values were very similar to those derived from the double reciprocal plots (0.36 μMol for room temperature and 0.17 μMol for 4°C). Even though the Kd was not definitive, it was still helpful in terms of setting concentration constraints for the extraction prototcol.

As mentioned previously, whey is a complex solution and factors such as more than one genetic variant of β-lactoglobulin, lactose, lipids, other proteins and micronutrients may influence the dynamics of the BSEAC system. In addition to the kinetic parameters, focus was also given to the separation efficiency of β-lactoglobulin from the whey by varying the protein concentration, pH and temperature (Figure 3.5, Tables 3.1 and 3.2).

The highest separation efficiency (78.28%) was obtained at 4°C at pH 4.6 when concentrations between 15.03 and 17.67 mg were applied (Figure 3.5). Below pH 3.5, the monomer is in rapid equilibrium with the dimer, between 3.7 and 5.2 higher order aggregates are formed (maximum self-association is at pH 4.5) and above pH 5.2 the dimeric form is predominant (11). The pH was not increased above 6.6 because β-lactoglobulin tends to slowly denature above pH 7.0 (15).

Tables 3.1 and 3.2 show the changes in efficiency (% recovery) as the concentration of applied β-lactoglobulin is varied at room temperature and 4°C, respectively. At both temperatures, the highest separation efficiency occurred when

14.0 to 15.0 mg of β-lactoglobulin was applied to the column, however the concentration of β-lactoglobulin in liquid whey is approximately 30 mg/ml. To optimize the separation of β-lactoglobulin the following options are possible: 1) increase the column size so that the amount of active ligand is increased, however, the required throughput of whey would be limited strictly by column size; 2) a combination of variant A and B may be used to covalently bind to the column matrix; and 3) the pH conditions could be varied to partially minimize the higher order aggregation. It was noted that β-lactoglobulin variant B had a higher recovery efficiency than variant A at 4°C. The tendency to tetramize is significantly less predominant in variant B (22), therefore, factors such as steric hindrance may have limited the separation of β-lactoglobulin variant A. At room temperature there is a higher degree of dissociation of the β-lactoglobulin and it was observed that in the higher temperature extraction, the two genetic variants were removed from the whey with very similar efficiencies. In addition, problems with column compaction and high back pressure at 4°C with β-lactoglobulin concentrations above 28.52 mg occurred which supports the theory that higher order aggregation may be inhibitory when whey is used as the substrate.

As with temperature, pH may also be used to modify the degree of association/dissociation in an attempt to maximize the separation. The pH would be the most practical variable to alter as higher temperatures may present problems in terms of microbial growth in the substrate.

3.4. CONCLUSIONS

In conclusion, Biospecific Subunit Exchange Affinity Chromatography was an effective tool for selectively removing β-lactoglobulin from whey. While traditional kinetic determinants were helpful in defining the system, separation efficiency was used to evaluate the BSEAC treatment of the whey. The BSEAC system relies on the natural ability of β-lactoglobulin to self associate, higher order aggregation may, however, reduce the efficiency of extraction when the total amount of β-lactoglogulin applied to the column is above 15.0-18.0 mg. Further studies would have to be conducted in terms of optimizing the exact conditions to maximize the separation efficiency by manipulating the degree of higher order aggregation.

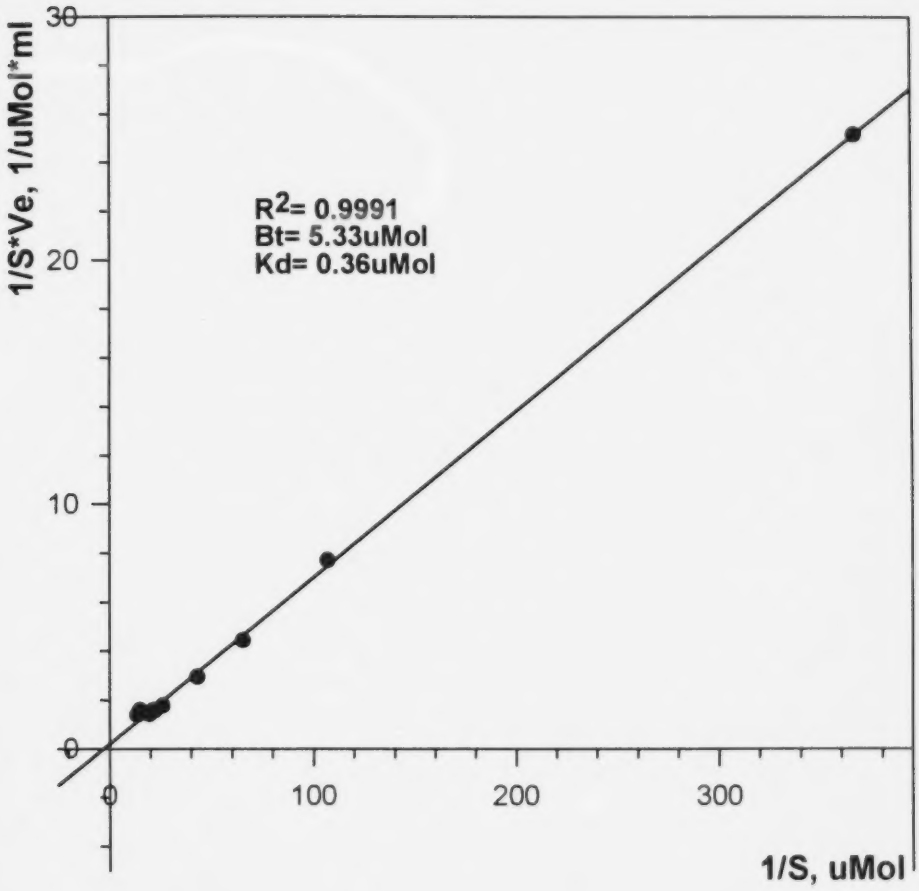

Figure 3.1 Double reciprocal plot of elution volume and substrate concentration for bioaffinity chromatographic data for β-lactoglobulin separated from whey at room temperature

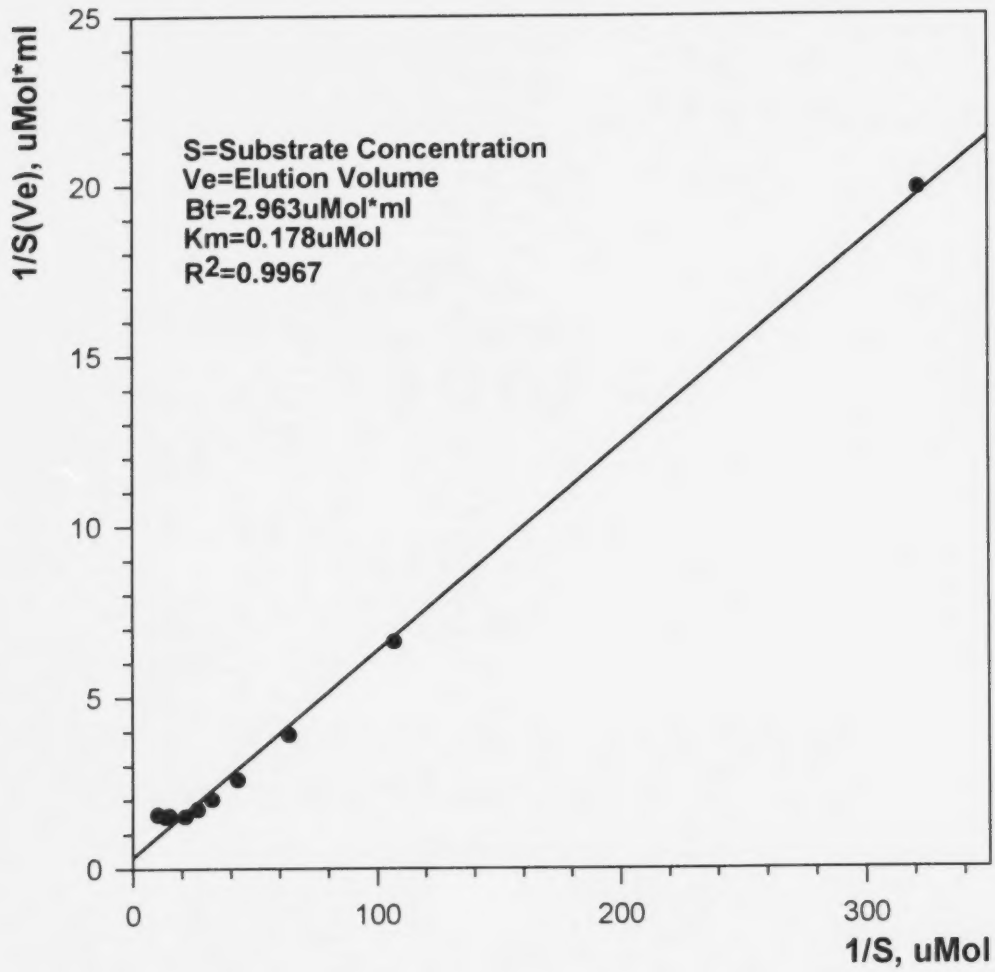

Figure 3.2 Double reciprocal plot of elution volume and substrate concentration for bioaffinity chromatographic data for β-lactoglobulin separated from whey at 4°C

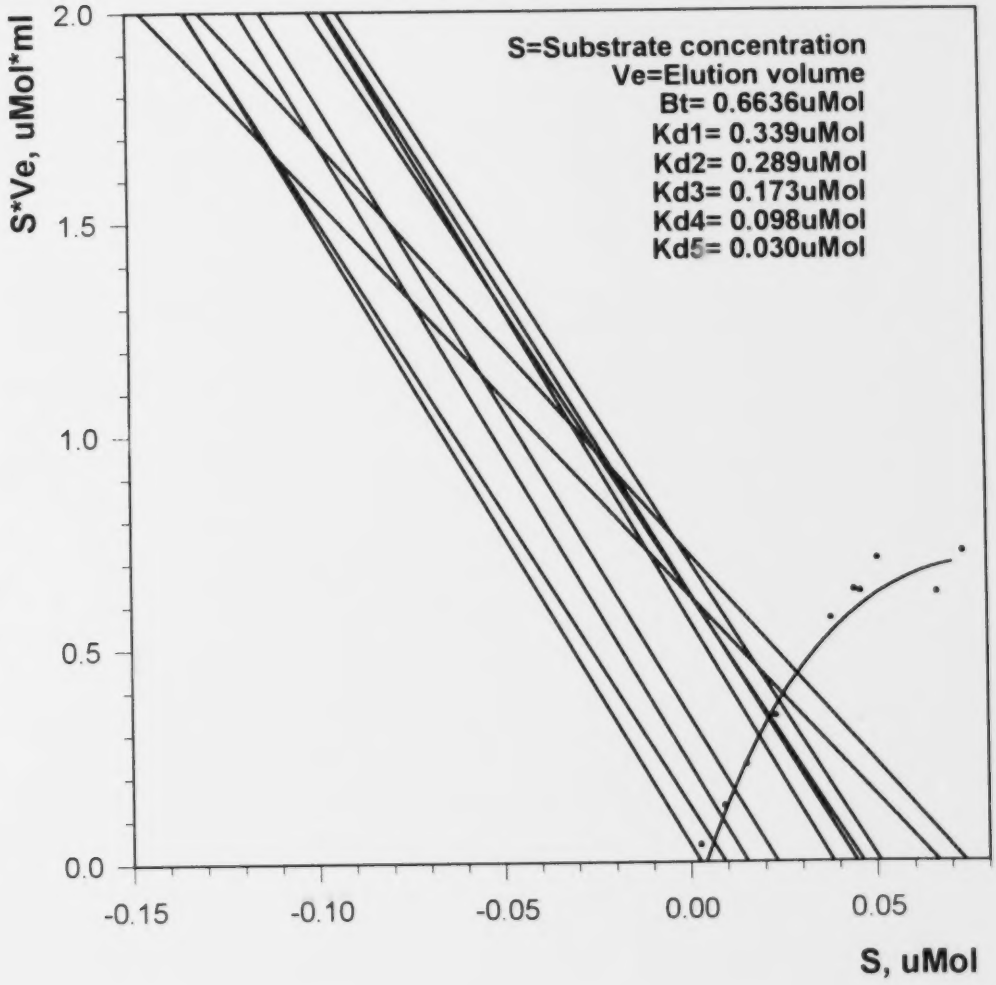

Figure 3.3 Graphical evaluation (direct linear plot) of Bt and Kd values for β-lactoglobulin measured by frontal affinity chromatographic methods in a bioaffinity system at room temperature

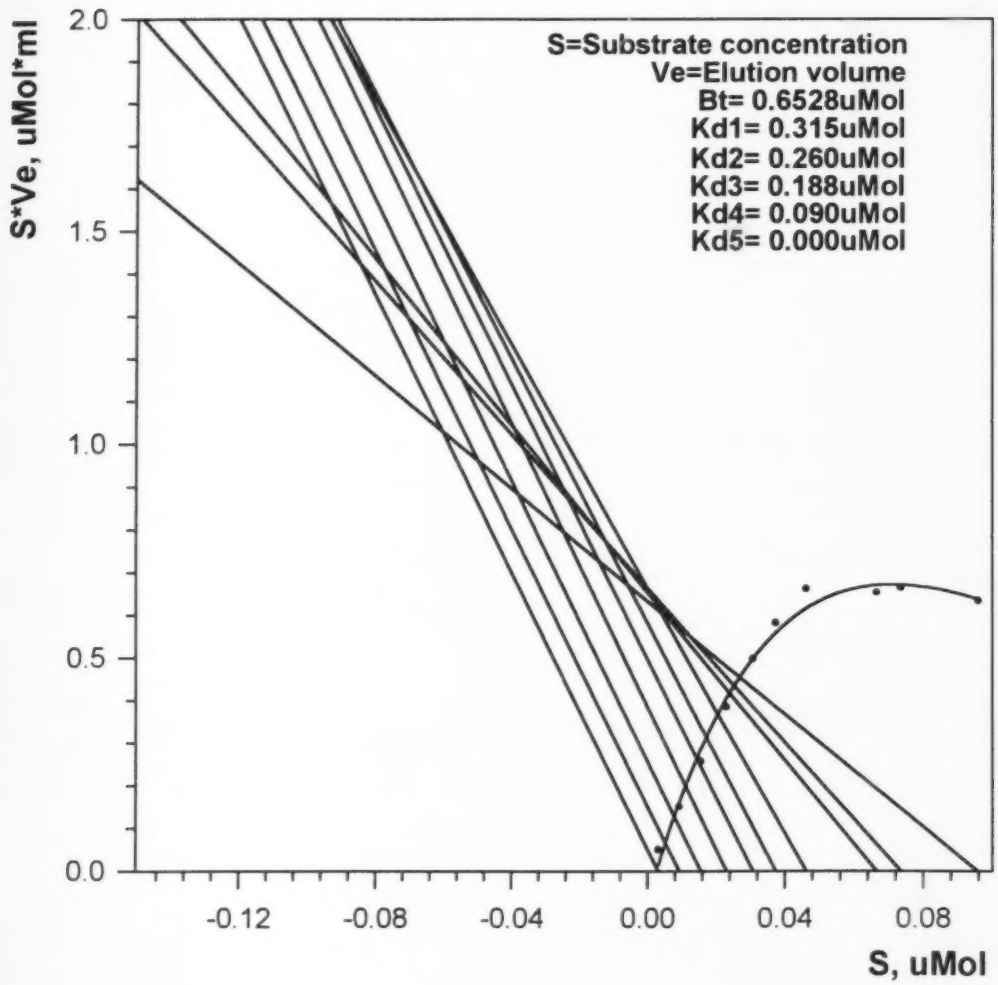

Figure 3.4 Graphical evaluation (direct linear plot) of Bt and Kd values for β-lactoglobulin measured by frontal affinity chromatographic methods in a bioaffinity system at 4°C

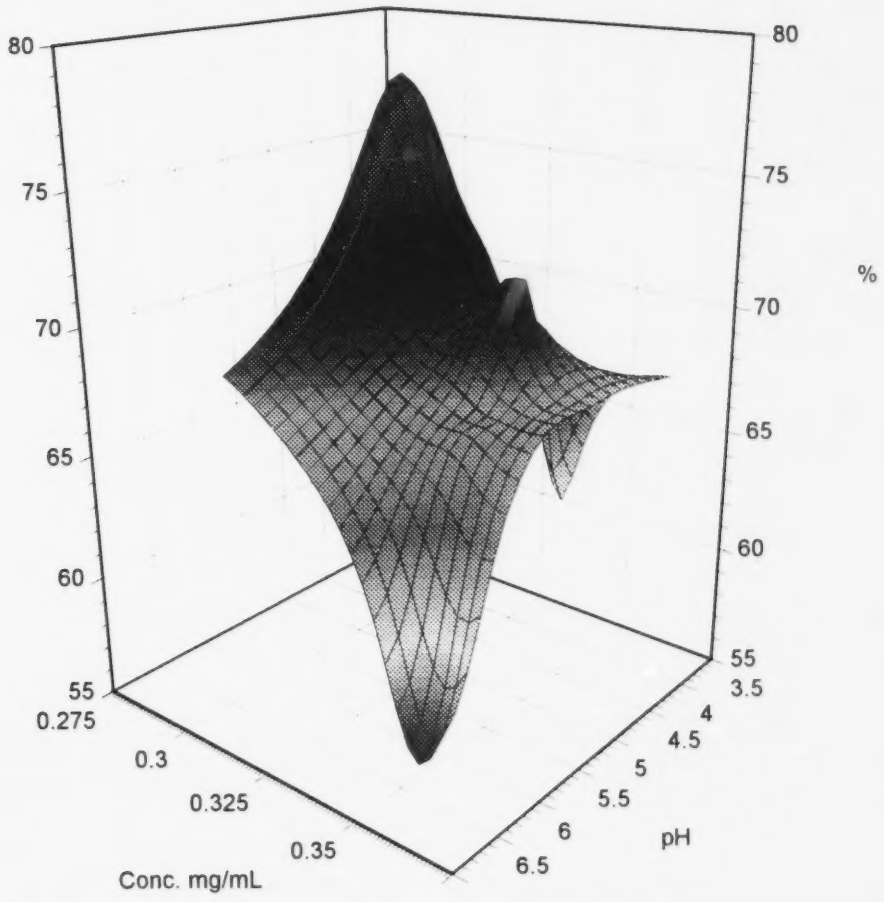

Figure 3.5 Separation of β-lactoglobulin from whey using subunit exchange bioaffinity chromatography under varied β-lactoglobulin concentrations and pH conditions at 4^0C

Table 3.1 Efficiency of β-lactoglobulin variant A and B extraction from whey at room temperature, pH 4.6 using bioaffinity chromatography

variant A & B applied (mg)	variant A applied (mg)	variant B applied (mg)	% variant A recovered	% variant B recovered	% total recovered
2.17	1.09	1.08	100.36	99.93	100.15
8.36	4.47	3.89	83.89	94.52	88.83
15.04	7.96	7.08	91.39	90.89	91.15
23.07	12.76	10.30	60.94	61.96	61.39
35.50	18.57	16.94	42.66	39.82	41.31
41.39	22.28	19.12	36.06	34.64	35.40
44.61	23.90	20.70	30.31	28.89	29.65
48.11	27.02	21.09	31.49	32.93	32.12
61.99	34.21	27.77	21.36	22.95	20.36
68.67	37.57	31.10	20.31	20.42	22.07

Table 3.2 Efficiency of β-lactoglobulin variant A and B extraction from whey at 4°C, pH 4.6 using bioaffinity chromatography

variant A & B applied (mg)	variant A applied (mg)	variant B applied (mg)	% variant A recovered	% variant B recovered	% total recovered
2.55	1.37	1.18	48.61	55.00	51.57
8.36	4.47	3.89	72.33	87.49	79.38
14.28	7.62	6.66	77.27	92.14	84.21
22.24	12.31	9.94	60.12	68.87	64.03
28.52	15.96	12.56	56.86	73.38	64.14
36.08	20.14	15.94	37.90	46.16	41.55
41.30	22.13	19.17	35.17	38.00	36.48
61.99	34.21	27.77	24.66	28.15	26.22
68.67	37.57	31.10	20.92	24.78	22.67
89.42	50.14	39.28	15.69	20.06	17.61

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CHAPTER 4

Aggregation Tendencies of β-lactoglobulin under varied pH and Temperature Conditions[1]

4.1. INTRODUCTION

Consumers demand more information regarding the nutritional and/or health potential of foods due to increased awareness regarding the relationship between food and health. There is a term which equates nutrition and pharmaceuticals - nutraceuticals. Nutraceuticals, according to the Foundation for Innovation in Medicine (Cranford, N.J.) are substances that may be considered a food or part of a food and provide medical or health benefits, including the prevention and treatment of disease.

Milk proteins are very versatile in terms of their physicochemical

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1 A version of this chapter has been accepted for publication. MacLeod & Ozimek 1995. Milchwissenschaft. **00** 000-000.

(1,2,3,4,5,6,7) and functional (7,8,9) characteristics which gives them potential not only as food ingredients but also as nutraceuticals. Properties of milk proteins and/or peptides that make them candidates for targeted application are: 1) biological activities such as opiate activity, antihypertension, immunomodulation, bioconversion of ions (Ca^{2+}) and antithrombotic activity (2); 2) protective (defense) functions (3); 3) regulatory roles (e.g. hormones and enzymes) (4,5,6); 4) antimicrobial activity and 5) transportation capabilities (e.g. calcium and retinol) (3, 10).

The behaviour of β-lactoglobulin under varied environmental conditions is not only the key to defining separation methodology but is also fundamental to elucidating physiological activity. The focus of this study was to examine the aggregation pattern of β-lactoglobulin under varied temperature and pH conditions. The aggregation tendencies were compared with separation efficiency results obtained using subunit exchange bioaffinity chromatography.

4.2. MATERIALS and METHODS

4.2.1. CHEMICALS

β-lactoglobulin variants A and B were crystallized products from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and the cheese whey was a hygroscopic

powder obtained from Alpha Milk Company (Red Deer, AB, Canada)

4.2.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The liquid chromatography system used consisted of the following Shimadzu components: SIL-6A auto injector unit, C-R6A Chromatopac integrator, SCL-6A system controller, LC-6A binary pumping system, SPD 10A dual channel detector (Fisher Scientific, Edmonton, Alberta, Canada).

4.2.3. EFFICIENCY OF REMOVAL OF β-LACTOGLOBULIN VARIANTS A and B FROM CHEESE WHEY

The affinity chromatographic system and methodology of MACLEOD and OZIMEK (12) was utilized to examine the removal (separation efficiency) of β-lactoglobulin from cheese whey under varied pH conditions at a constant temperature (4°C).

4.2.3.1 SAMPLE PREPARATION.

Whey powder was dissolved in water and centrifuged at 10,000 X g for 25 minutes at room temperature to remove any particulate matter. Sodium acetate and acetic acid were used to adjust the pH of the whey solution and the associating buffer (variations in pH between 3.5 and 5.2. The salt concentration was kept

constant in all of the trials (0.1 M Na-acetate). It was necessary to dissolve the whey in water rather than associating buffer directly, as the β-lactoglobulin would aggregate and be lost during centrifugation; the loss was pH dependent. The concentration of β-lactoglobulin applied to the column in the various whey preparations was verified using the methodology of MACLEOD et al.(16).

4.2.3.2. SEPARATION DYNAMICS.

The efficiency of β-lactoglobulin variants A and B extraction from whey under varied pH conditions was calculated using the following: Separation efficiency (SE) = (mg β-lactoglobulin recovered/mg β-lactoglobulin applied) x 100.

4.2.4. DETERMINATION OF HIGHER ORDER AGGREGATION

The HPLC system previously discussed was used with the exception of the column; a Pharmacia (Uppsala, Sweden) Phenyl-Superose 5/5 HR was utilized. The hydrophobic interaction chromatographic (HIC) separation was performed as described by GRINBERG et al. (17) with the following modifications: 1) the choice of hydrophobic column i.e. phenyl versus methyl polyether phase and 2) the mobile and elution buffers were modified to accommodate the increased hydrophobicity of the Phenyl-Superose. The mobile phase A consisted of 0.05 Tris-HCl and 1.27 M ammonium sulfate, pH 4.6 and the elution buffer B was made up of 0.05 M Tris-HCl in 35% acetonitrile. The running program was a linear gradient; 100% A to 100%

B in 17 minutes with a flow rate of 0.5 ml/min and a total run time of 32 minutes. The column was cleaned and equilibriated before each run. The samples, β-lactoglobulin A, β-lactoglobulin B or a combination of A and B (total protein concentration in all samples was 10 mg/ml) were dissolved in buffer A and prepared as per the procedures outlined in the HPLC section. The elution profile was monitored using a wavelength of 280 nm.

4.2.5. PARTICLE SIZE DISTRIBUTION

Particle size distribution as influenced by pH and temperature was monitored by light scattering using a Brookhaven Instruments Corporation BI-90 (Holtsville, N.Y., U.S.A.). All experimental runs were carried out in triplicate using the following equipment parameters: 1) test duration of 5000 cycles; 2) viscosity setting of 0.933cp; and 3) dust cut-off of 30 nm.

4.2.5.1. SAMPLE PREPARATION

β-lactoglobulin variant A was dissolved in the associating buffer (0.1 M Na-acetate) described by MACLEOD and OZIMEK (12). The pH was varied between 3.5 and 6.6 (3.5, 3.7, 4.2, 4.6, 4.9, 5.2, 5.6 and 6.6) for both testing temperatures (4°C and 23°C). The samples were dissolved and allowed to stand for 1 h prior to testing. It should be noted that the time interval (standing time) was too short for the proteins to reach complete equilibrium. Complete equilibration might require up to several days to be achieved but would not represent the proteins during

extraction (i.e. during biospecific subunit exchange affinity chromatography) where the total sample preparation and run time is 1.5 h. However, the conditions used in this study were consistent for all protein substrates so that relative differences could be monitored. The sampling was conducted so that each set of triplicates was produced in a randomized fashion in order to diminish the time dependency of the aggregation variable. Blank buffers were screened prior to running each sample, no buffer interference was observed.

4.3. RESULTS and DISCUSSION

The ability of β-lactoglobulin to form aggregates through self-association is due to: 1) specific recognition and 2) stabilizing forces such as hydrogen bonds, hydrophobic and electrostatic interactions. Due to the stabilizing forces, the reaction can be manipulated using: 1) pH; 2) temperature; 3) ionic strength and 4) dielectric constants. Aggregation tendencies of β-lactoglobulin variant A and B as well as equimolar concentrations of the two variants were examined using hydrophobic interaction chromatography (HIC) (17). The retention times obtained when temperature and pH were varied are given in Table 4.1.

At pH 2.0, when β-lactoglobulin experiences a high degree of dissociation, the retention times did not appear temperature dependent, 32.07 to 34.39 minutes at room temperature and 31.93 to 33.86 minutes for 4°C (variants A and B, respectively) (11, 17). The sample containing variants A and B in combination,

eluted with the peaks appearing at approximately the same time as the variant A sample (32.41 min, RT and 31.86 min at 4°C).

At pH 4.6, where there is a high degree of association in β-lactoglobulin, there were only small changes in the retention patterns between the variants as well as between the two experimental temperatures.

At pH 7.5, β-lactoglobulin is predominantly in the dimer form and at the colder temperature (4°C) the elution time was significantly faster than at room temperature. The HIC profiles gave indication that changes were occurring between the two temperatures at the varied pH's, however, the trend in aggregation was not fully elucidated.

Particle size distribution (PSD) utilizing dynamic light scattering technology was used to determine the aggregate size profiles of β-lactoglobulin under various conditions of pH and temperature. The PSD patterns for β-lactoglobulin A at 4°C and room temperature are shown in Figure 4.1.

A temperature variation in the PSD patterns was observed with the differences being more marked at the pH's above 4.2. The self-association phenomena is thought to be due to pH induced shifts in the side-chain charge. At pH 3.5 the monomeric and dimeric forms of β-lactoglobulin are thought to be in equilibrium (11). It is hypothesized that at pH 4.2, the pH at which the PSD is the smallest, that the equilibrium is in transition, or in other words, there is a shift in side-chain charges. The maximum particle size (340 nm) was observed at the

isoelectric point of β-lactoglobulin (pH 5.2) indicating the most higher order aggregation. It may be assumed that these changes are related to structural changes in the protein at its isoelectric point.

In Table 42, the efficiency of removal of β-lactoglobulin from cheese whey under varied pH conditions using subunit exchange bioaffinity chromatography at a constant temperature of 4°C is given. Comparing Figure 1 and Table 2, there is a considerable increase in particle size at pH >4.6 along with declining separation efficiencies. In addition to the above, MACLEOD and OZIMEK (unpublished observations, 1994) observed that between pH 4.6 and 5.2, there was an increase in back pressure which resulted in column compaction, further indicating higher order aggregation. To increase the separation efficiency from 78.28 % to a theoretical 100.0 % one solution may entail reselection of the matrix; one that would allow for separation of particles in excess of 340 nm.

4.4. CONCLUSIONS

In conclusion, Biospecific Subunit Exchange Affinity Chromatography (BSEAC) is an effective tool for selectively removing β-lactoglobulin from whey, however improvements on the overall separation efficiency can be made. Using both the hydrophobic interaction chromatographic data and the particle size distribution results, it was concluded that the optimum separation at pH 4.6 was probably due to column inadequacies rather than inherent properties associated

with β-lactoglobulin. It was proposed that the separation of β-lactoglobulin could be enhanced if the separation was run at a pH of 5.2 and a temperature of 4°C. The isoelectric point of β-lactoglobulin (pH 5.2), the point of the highest degree of structural change and hence the highest degree of aggregation would allow for more complete/efficient extraction of the protein from whey. A column with a larger molecular weight cut-off would increase the total amount of protein that could be initially applied to the column.

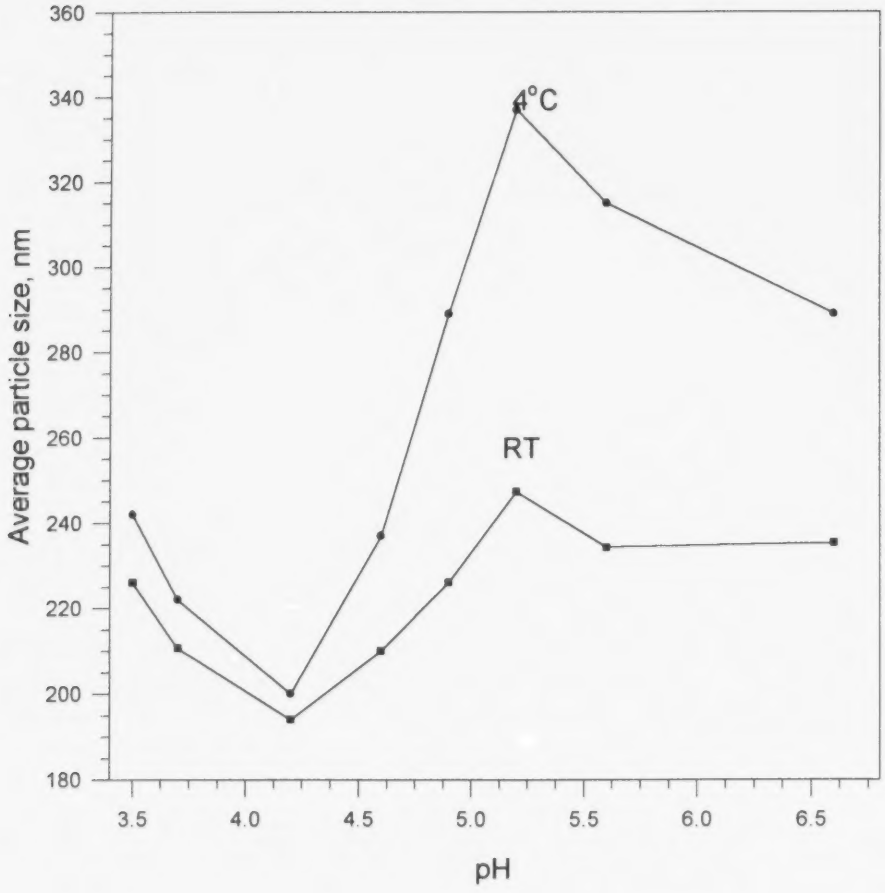

Figure 4.1. Particle size distribution patterns for β-lactoglobulin variant A obtained using light scattering technology with pH and temperature as the experimental variables

Table 4.1 The retention times obtained from hydrophobic interaction chromatography of β-lactoglobulin variants A, B and equimolar mixtures of A and B (all samples contained a total of 10 mg/ml of protein) when temperature and pH were varied

	Variant A	Variant B	Variants A and B
4°C		Elution time (minutes)	
pH 2.0	31.92	33.86	31.86
pH 4.6	24.45 27.15	24.09 27.00	24.16 27.08
pH 7.5	21.60	22.40	24.54
Ambient temperature			
pH 2.0	32.06	34.39	32.41
pH 4.6	24.04 25.85	23.62 26.74	23.75 26.15
pH 7.5	25.20	28.42	25.94 28.70

Table 4.2 Effect of pH on the efficiency of β-lactoglobulin extraction from whey using subunit exchange bioaffinity chromatography

pH	β -lactoglobulin applied	β -lactoglobulin recovered	% recovery
3.5	15.95 ± 0	5.80 ± 0.37	36.40 ± 2.34
3.7	17.83 ± 0	10.48 ± 0.01	58.81 ± 0.01
4.2	17.18 ± 1.44	12.17 ± 1.24	70.83 ± 4.72
4.6	15.91 ± 1.52	12.46 ± 1.21	78.28 ± 0.48
4.9	17.83 ± 0	12.61 ± 0.35	70.79 ± 1.94
5.2	18.88 ± 0	12.75 ± 0.37	67.53 ± 1.98
5.6	18.16 ± 0	12.22 ± 0.52	67.31 ± 2.86
6.6	18.56 ± 0	10.83 ± 0.20	58.28 ± 1.05

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CHAPTER 5

Binding of retinoic acid to β-lactogloblin variants A and B: Effect of peptic and tryptic digestion on the protein ligand complex[1].

5.1. INTRODUCTION

The structure of β-lactoglobulin as it relates to functional characteristics such as retinoid binding capabilities has been extensively studied (1,2,3,4,5,6,7,8,9,10,11,12). A crystallographic examination conducted by Papiz et al. (1) determined the essential conformational structure of the protein and proposed that the β-barrel was remarkably similar to the binding site of plasma retinol-binding protein (1). Cho et al. (6) further substantiated that retinol binding

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1 A version of this chapter has been accepted for publication. MacLeod, Fedio & Ozimek 1995. Milchwissenschaft. **00** 000-000.

occurs at the evolutionary conserved calyx-shaped interior cavity of β-lactoglobulin. Structural changes as influenced by pH, temperature, ionic strength, dielectric constant, etc. have all been shown to affect the binding ability of β-lactoglobulin (7,8,9,11).

Physicochemical activity of fat soluble vitamins are often mediated through protein interaction which: 1) increases availability; 2) decreases oxidative destruction and 3) reduces cytotoxicity (11,13,14). Enzymes such as pepsin and trypsin which are found in the stomach and upper gastrointestinal tract (respectively) are part of the digestive process which change protein structure through cleavage (15). The ability of the protein to interact with the vitamin as well as the effect of enzymatic digestion on the complex are key to understanding the bioavailability and physicochemical significance of the protein/ligand structure.

The primary objective of the present work was to evaluate the binding kinetics of β-lactoglobulin with retinoic acid and study the effect of digestive enzymes on the protein and protein/ligand complex.

5.2. MATERIALS and METHODS

5.2.1. CHEMICALS

β-lactoglobulin genetic variants A and B and retinoic acid (all trans) were

crystallized products from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lyophilized, dialyzed, crystallized powder of bovine pancreas trypsin and Type II-S soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pepsin was obtained from Fisher Scientific (Edmonton, AB, Canada). Activated Carbon powder (Darco G-60) was a product of Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

5.2.2 CHARCOAL TREATMENT

Bound lipids were removed from the β-lactoglobulin variants A and B using the method of Chen (16). Darco G-60 activated carbon was first prepared by washing with milliQ water then drying at room temperature. 1.5 g of β-lactoglobulin variants A and B were dissolved in 15.0 ml of milliQ water and then combined with 0.75 g of washed carbon. The pH of the protein/carbon mixture was adjusted to pH 3.0 with 0.2 N HCl before placing in an ice water bath (continuous stirring) for 1 hr. Centrifugation was carried out using a Beckman JA-20 fixed angle rotor at 20,400 x g (13,000 rpm) for 20 min at 2^{0}C. The supernatant was filtered through a .2 μm cellulose acetate millipore filter prior to adjusting the pH to 7.0 with 0.2 N sodium hydroxide. The resulting solution was freeze dried.

5.2.3 FLUORESCENCE SPECTROSCOPY

The fluorescence measurements were carried out on a Perkin Elmer Luminescence Spectrometer LS 50B using an excitation wavelength of 296 nm and an emission wavelength of 310 nm (the slit width for both the excitation and emission wavelengths were set at 2.5 nm).

5.2.3.1. β-LACTOGLOBULIN TITRATION

Using the methods of Cogan et al. (7) and Dufour and Haertle (17) the following procedures were used to titrate the β-lactoglobulin and retinoic acid: 2 ml of β-lactoglobulin solution (concentration of 1 μM) were placed in a cuvette, incremental amounts (2 μL) of ligand (retinoic acid in ethanol, .15 mM) was injected into the protein solution with a Socorex micropipette. The protein/ligand solution was mixed and allowed to stand for 5 min prior to fluorescence measurement. For each titration point, a buffer/ligand blank was prepared and treated in the same manner as the sample. All titration points were run in triplicate.

5.2.4. DETERMINATION OF APPARENT DISSOCIATION CONSTANTS

The dissociation constants (Kd) were examined using two different graphical methods: 1) a regression method (7,17) and 2) a direct linear plot (18). The regression data was generated by plotting ($P_0 \cdot a$) vs. B{a/(1-a)}, a straight line was

obtained with an intercept of Kd/n and a slope of 1/n; where Kd is the apparent dissociation constant; n is the apparent molar ratio of ligand/protein at saturation; P_0 is the total protein concentration and B is the total ligand concentration a is defined as the fraction of unoccupied binding sites on the protein while n is the fraction of occupied sites $a = (F-F_{min})/(F_0-F_{min})$ where F represents the fluorescence intensity at a certain B (corrections are made for the blank), F_{min} represents the fluorescence intensity upon saturation of the protein and F_0 is the initial fluorescence intensity.(17).

The direct linear plotting method requires that fluorescence (corrected for blank) is plotted directly against the ligand concentration. Using this method, no mathematical manipulation of the data is required as the Kd is obtained directly from the intersection points on the plot.

5.2.5 ENZYMATIC DIGESTION

The effect of enzymatic digestion on β-lactoglobulin and the β-lactoglobulin/retinoic acid complex was investigated.

5.2.5.1. PEPTIC HYDROLYSIS

The method of Ozimek (unpublished) was used to carry out the hydrolytic examination. The protocol was as follows: 10 mg/ml of β-lactoglobulin dissolved

in 0.075 N HCl (pH 2.0) with an addition of 8.2 I.U. of pepsin (5.43 mg). The solution was held for 4 hr and 37^{0}C in a New Brunswick Scientific Controlled Environment Incubator Shaker with continuous sampling at 30 min intervals. The hydrolysis was stopped in each sample using 3 M Tris-HCl (pH 8.8); the Tris -HCl additions equalled 10 % of the total sample volume. The digestion study was carried out in triplicate.

5.2.5.2. TRYPSIN HYDROLYSIS

The hydrolysis buffer outlined in the Worthington manual (19) (0.05 M Tris-HCl, pH 8.1 with 0.00115 M $CaCl_2$) was utilized as the base solution. To the solution 10 mg/ml of β-lactoglobulin was added along with enough trypsin to accomplish a 1:500 enzyme/protein ratio. The digestion was carried out in a New Brunswick Scientific Controlled Environment Incubator Shaker for 4 hrs at 37^{0}C with sampling occurring at 30 min intervals. The enzymatic reaction was stopped in each sample using trypsin inhibitor. Tryptic hydrolysis was carried out for the following protein and protein/ligand combinations: 1) native β-lactoglobulin variant A; 2) charcoal treated β-lactoglobulin variant A; 3) native β-lactoglobulin variant A bound to retinoic acid and 4) charcoal treated β-lactoglobulin variant A bound to retinoic acid. All runs were performed in triplicate.

5.2.5.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) HYDROLYSIS EVALUATION

The liquid chromatography system used consisted of the following Shimadzu

components: SIL-6A auto injector unit, C-R6A Chromatopac integrator, SCL-6A system controller, LC-6A binary pumping system, SPD 10A dual channel detector (Fisher Scientific, Edmonton, Alberta, Canada). The procedures outlined by Sitohy et al. (20) were the basis for the evaluation of the protein degradation. A Nucleosil C_{18} column (Phenomenex, Torrance, CA) was equilibrated in solvent A (0.15 M NaCl/HCl, pH 2.5), and elution was obtained using a linear gradient from solvent A to solvent B (60 % acetonitrile and 40 % solvent A) in 32 min. The run temperature was 40^{0}C at a flow rate of 1 ml/min and uv absorbance was monitored at 214 nm.

5.3. RESULTS AND DISCUSSION

Kinetic parameters such as dissociation constant (Kd) and apparent molar ratio of ligand/protein (n) were used to define the binding of β-lactoglobulin variants A and B to retinoic acid. The results from fluorometric quenching interpreted by both mathematical and graphical means are represented in Figures 5.1 through 5.4. The direct linear plotting results for β-lactoglobulin variants A and B are given in Figures 5.1 and 5.3, respectively (18). The Kd values obtained from the direct linear plots were 1.96 X 10^{-7} M and 1.75 X $^{-7}$10 M for variants A and B, respectively. In contrast, the Kd for variant A was 1.0 X 10^{-8} M and the variant B dissociation constant was 2.68 X 10^{-10} M (Figures 5.2 and 5.4) using regression analysis. The Kd values for the direct linear plots were more consistent with other

published observations (6). Using all data points in the experiment, regression coefficients of r^2 = .8905 and .8935 were obtained for β-lactoglobulin variants A and B (respectively). It was observed that the data points fall into a curvilinear rather than straight line pattern. Plotting retinoic concentration versus absorbance with a constant protein concentration, it was observed that the curve actually represents the saturation point of the protein. If the curve or saturation point is eliminated from the regression line the Kd will be misrepresented (i.e. give indication of higher affinity)

The direct plotting method of Eisenthal and Cornish-Bowden (18), deals with the data in observation space creating a regression line for each individual observation. The Kd is then determined using the mean of the intersecting points for the individual lines eliminating errors that occur at the extremes (i.e. saturation). The linearization of data either over or under estimates the binding ratio of the protein to ligand complex. In the case of our data, n values for the regression presentation were n = 0.46 for variant A and 0.47 for variant B. The saturation point in the direct linear plot was taken to be n as it was the absorbance that was constant despite any further additions of ligand i.e. n = 1.1 for both variants A and B. Reported n values are between 0.79 and 1.0 for β-lactoglobulin/retinoid binding studies; variations in experimental protocol which include data manipulation may explain the inconsistencies in terms of molar protein/ligand binding ratio.

The potential application of β-lactoglobulin as a retinoid carrier can not be

fully elucidated without determining its digestibility in the gastrointestinal tract. HPLC protein/peptide mapping showed that β-lactoglobulin was resistant to peptic hydrolysis even after 4 hours of incubation at 37^{0}C, pH 2.0 (Fig 5.5). Pepsin splits proteins into proteose and peptone fractions which are comparatively large peptide molecules. β-lactoglobulin has been shown to be resistant to acidic peptic hydrolysis after 2 hours of digestion (15). The chromatograms obtained after 1 and 4 hrs of digestion (Fig 5.5) show differences in small peak height, however, the relative size of the β-lactoglobulin peak remains constant.

The effect of retinoic acid binding on tryptic hydrolysis of β-lactoglobulin was examined (Table 5.1). When native β-lactoglobulin A and defatted β-lactoglobulin A were exposed to trypsin for 4 hours considerable digestion occurred (92.56% and 92.90%, respectively). In contrast, when bound to retinoic acid, both the native and the charcoal treated β-lactoglobulin test groups demonstrated increased resistance to trypsin (i.e. only 17.63% and 23.78% digestion, respectively) indicating that hydrophobic molecules such as retinoic acid may serve to protect β-lactoglobulin from specific digestive processes.

5.4. CONCLUSIONS

Based on fluorometric quenching experiments it can be shown that β-lactoglobulin binds retinoic acid. The molar binding ratio for the protein/ligand

interaction is dependent on the method used to evaluate the kinetic parameters i.e. direct linear plot versus regression analysis. It was concluded that the direct linear plot of Eisenthal and Cornish-Bowden (18) is a more appropriate method for data analysis as it deals with the data in observation space eliminating transformation errors. According to the direct linear graphs the molar bind ratio for protein to ligand (n) was 1: 1.1 which indicates that 1 molecule of protein bind 1 molecule of retinoic acid. In addition, the apparent dissociation constants were dependent on the analytical interpretation with the direct linear plot giving affinities which were closer to other published data i.e. $Kd = 1.96 \times 10^{-7}$ for β-lactoglobulin variant A and 1.75×10^{-7} for variant B.

The retinoic bound β-lactoglobulin was examined for its ability to withstand peptic and tryptic digestion at $37^{0}C$ (pH 2.0 and 8.1, respectively). Although resistant to pepsin digestion, β-lactoglobulin was highly susceptible to digestion by trypsin (in excess of 90% was digested). When β-lactoglobulin was bound to retinoic acid there was a significant reduction in its digestibility (less than 25% of the protein was broken down) indicating that hydrophobic molecules such as retinoic acid may serve to protect β-lactoglobulin from specific digestive processes.

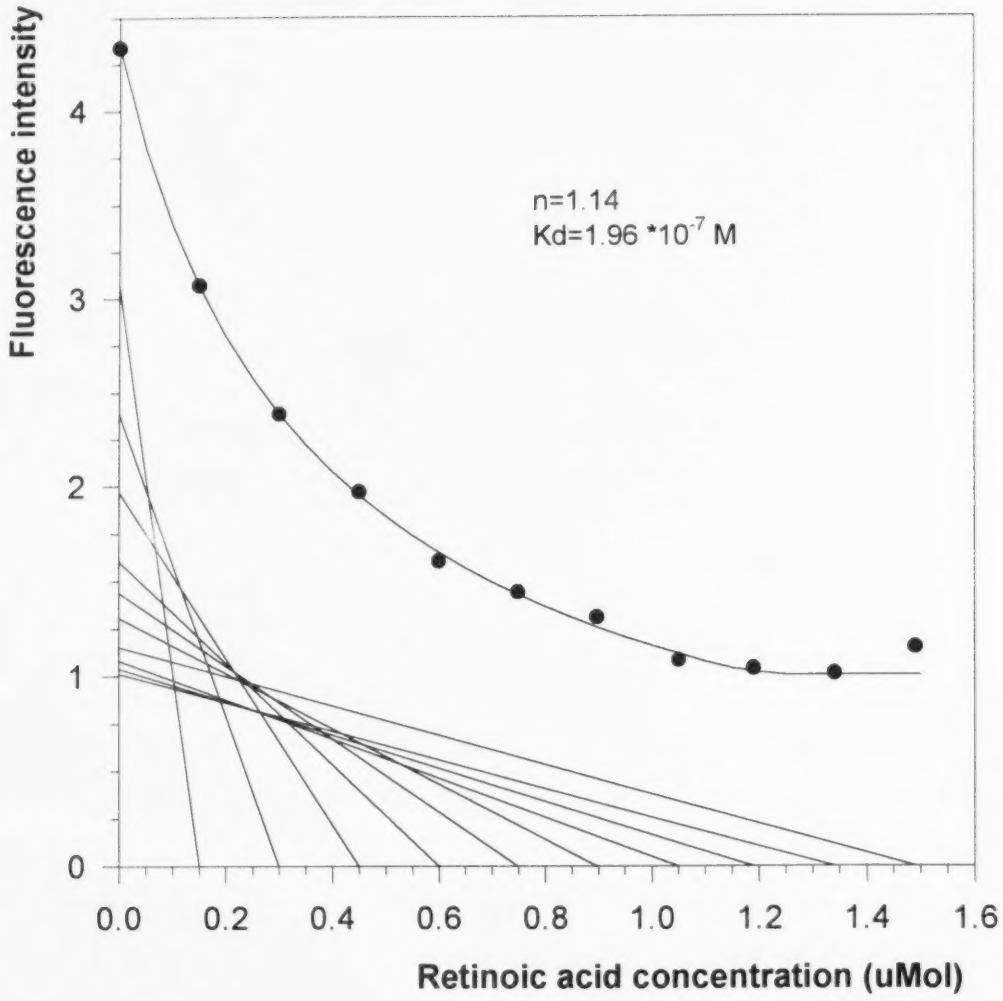

Figure 5.1. β-lactoglobulin variant A and retinoic acid protein/ligand binding parameters (apparent dissociation constant, Kd and molar binding ratio, n) as determined using a direct linear plot.

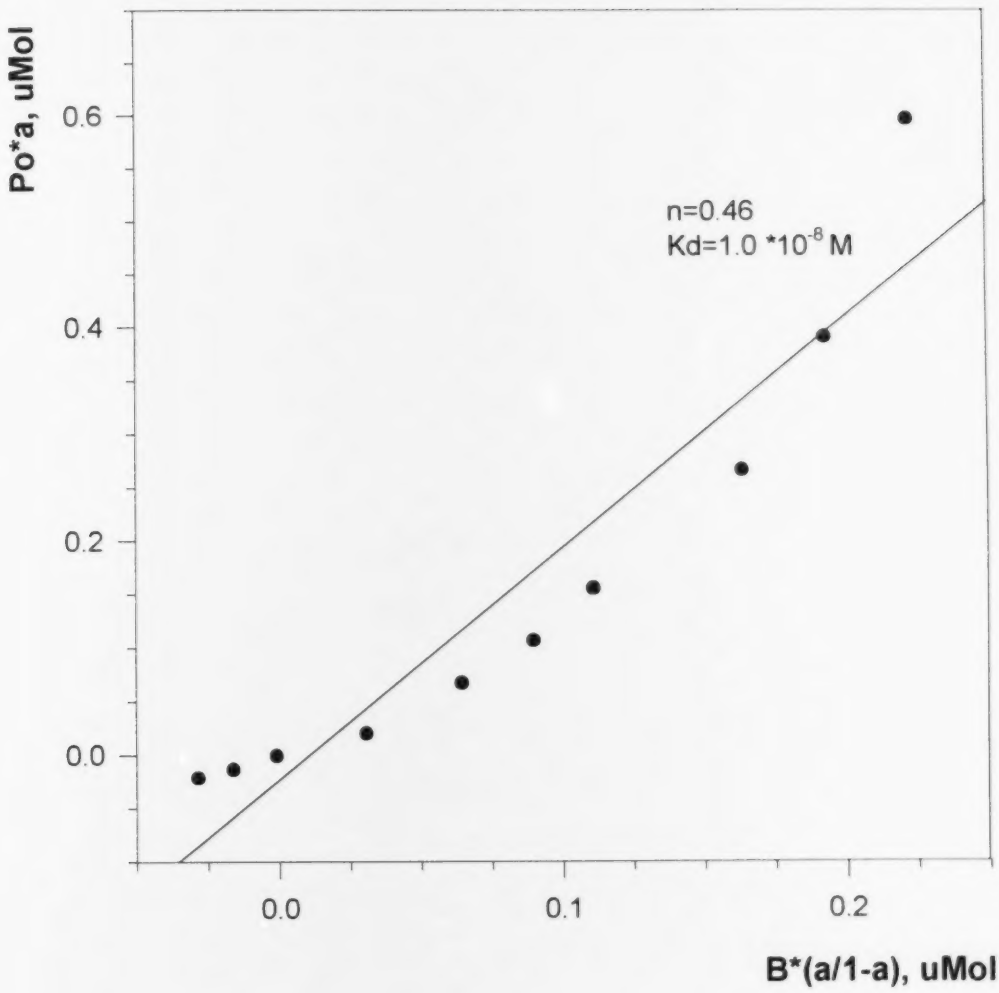

Figure 5.2. Apparent dissociation constant (Kd) and molar binding ratio (n) for β-lactoglobulin variant A and retinoic acid as determined using regression analysis.

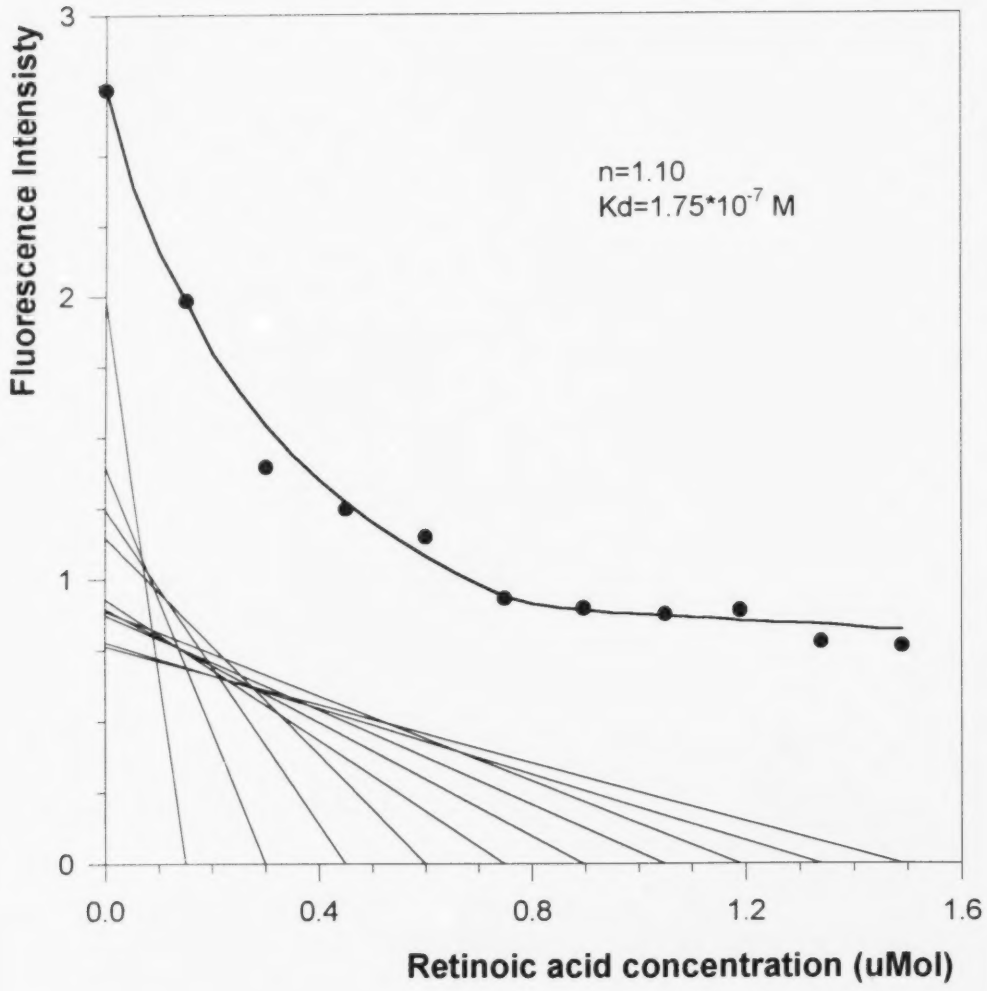

Figure 5.3. β-lactoglobulin variant B and retinoic acid protein/ligand binding parameters (apparent dissociation constant, Kd and molar binding ratio, n) as determined using a direct linear plot.

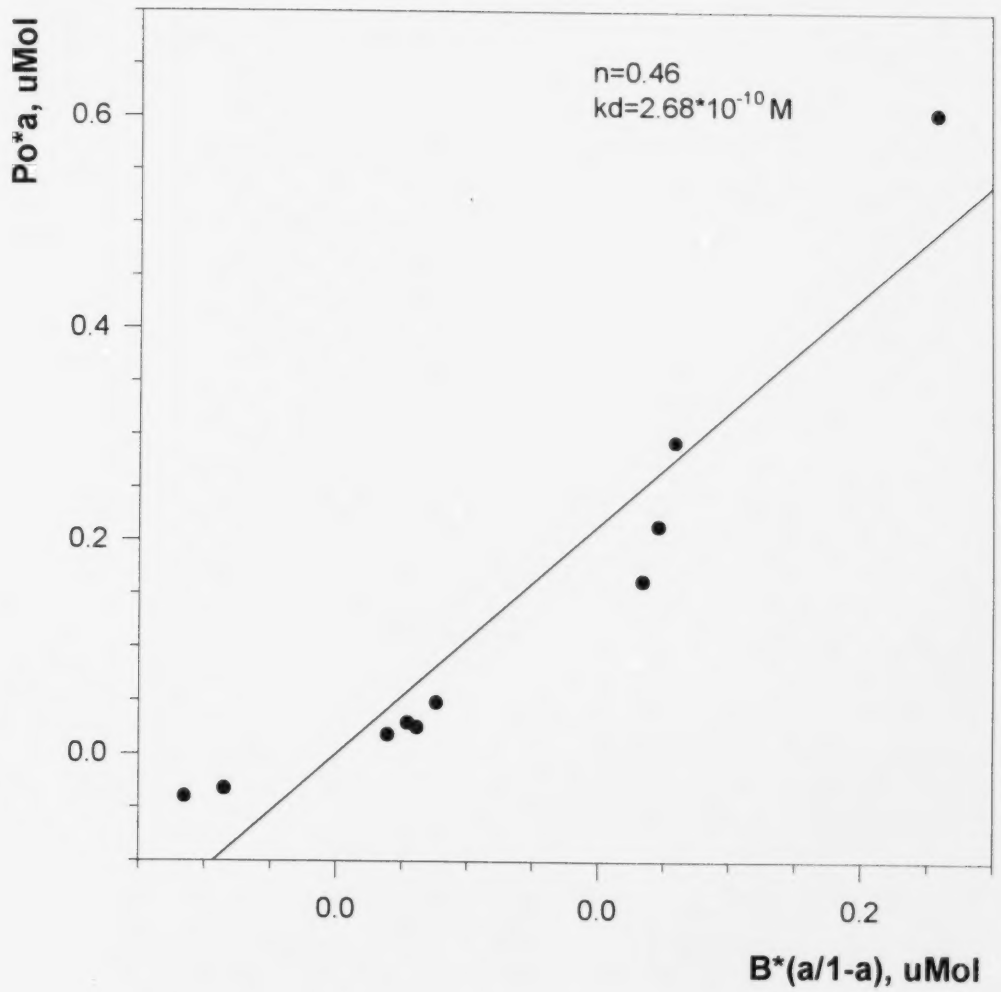

Figure 5.4. Apparent dissociation constant (Kd) and molar binding ratio (n) for β-lactoglobulin variant B and retinoic acid as determined using regression analysis.

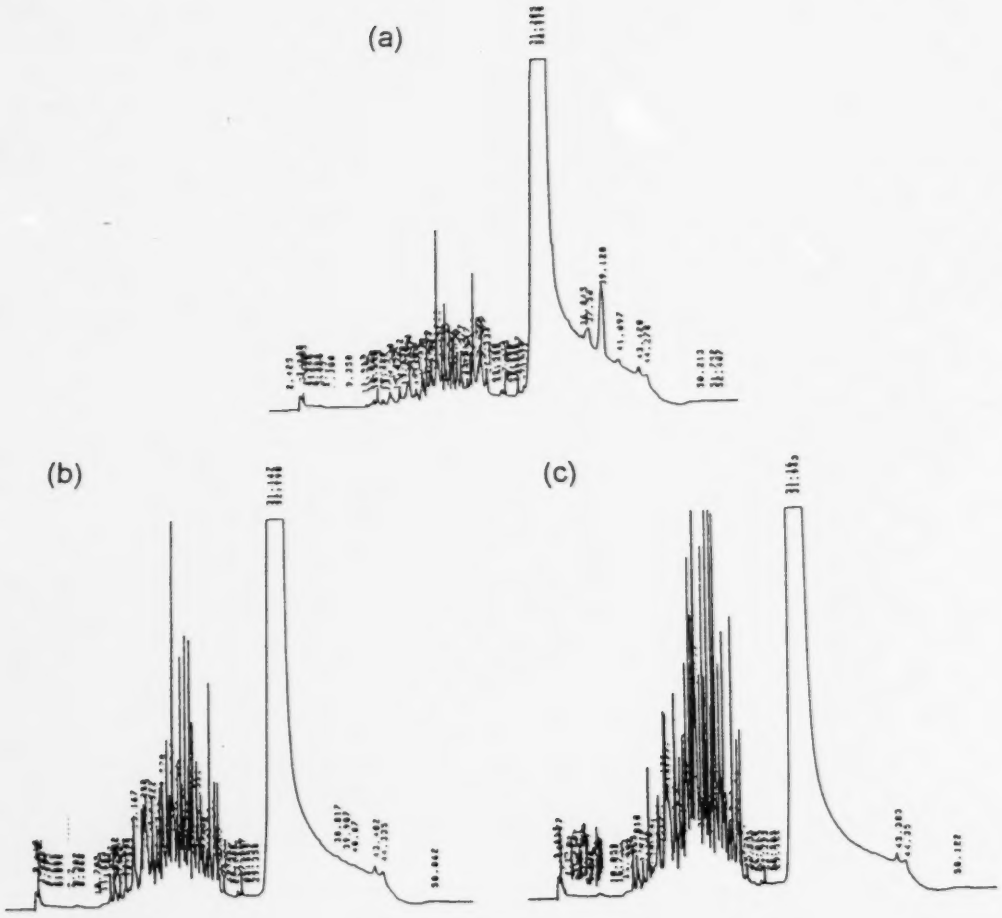

Figure 5.5. Reverse phase High Performance Liquid Chromatography peptide profiles of β-lactoglobulin A exposed to peptic enzyme at 37°C, pH 2.0 for 0 min (a), 2 hrs (b) and 4 hrs (c).

Table 5.1 Effect of trypsin on native, charcoal treated and retinoic acid bound β-lactoglobulin variant A at 37°C, pH 8.1 as determined by reverse phase high performance liquid chromatography

Substrate	% digested after 4 hours
Native β-lactoglobulin A & retinoic acid complex	17.63 ± 4.50
Native β-lactoglobulin A	92.56 ± 0.78
Charcoal treated β-lactoglobulin A & retinoic acid complex	23.78 ± 3.62
Charcoal treated β-lactoglobulin A	92.90 ± 2.40

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CHAPTER 6

Concluding Remarks

6.1 SUMMARY OF RESEARCH FINDINGS

Studies were carried out to evaluate the separation of β-lactoglobulin A by biospecific subunit exchange affinity chromotography (BSEAC) and to measure the kinetics of the reaction. It was shown by a direct linear plot that there was more than one dissociation constant (Kd) which indicated not only monomer/monomer interaction but also higher order aggregation during self-association. The Kd values were 104 μMol and 120 μMol for the dissociation of formed dimer and higher order aggregates, respectively. The higher order aggregation during BSEAC was

confirmed by hydrophobic interaction chromoatography (HIC).

Additional work was carried out selectively isolating β-lactoglobulin from cheese whey using biospecific subunit exchange affinity chromatography (BSEAC) under varied environmental conditions that included temperature, pH and protein concentration. Isolation of β-lactoglobulin was achieved by the interaction between β-lactoglobulin covalently bound to a Sepharose S-CNBr-4B matrix and the protein in solution. A direct linear plotting method showed the Bt values to be temperature, pH and concentration independent. The Bt values for room temperature and 4°C were 0.65 and 0.66 μMol, respectively. The average Kd values for the cheese whey extraction model were 0.36 and 0.17 μMol at room temperature and 4°C, respectively. The highest separation efficiencies achieved were 91.15% and 84.21% for room temperature and 4°C, respectively with the initial β-lactoglobulin loading in the 15.0 mg range. It has been shown that β-lactoglobulin can be separated from cheese whey by BSEAC. This process may result in the commercial manufacture of β-lactoglobulin reduced whey protein concentrates as well as a native, pure β-lactoglobulin fraction. Both products may have specific nutritional/pharmaceutical applications.

Using hydrophobic interaction chromatography (HIC) and particle size distribution (PSD) aggregation phenomena, the basis for the selective extraction of β-lactoglobulin, was examined. While HIC proved to be inconclusive, a general pattern was obtained. Particle Size Distribution is a light scattering technique

which can be utilized to determine average particle size (nm) suspended in a solution. A distinct profile evolved when particle size was plotted against pH for the two experimental temperatures. While β-lactoglobulin has a tendency to tetramize between pH 3.7 and 5.2, it was observed that the highest degree of aggregation was at the isoelectric point (pH 5.2). At pH 4.6 the aggregates had an average size of 240 nm while at pH 5.2 the average was 340 nm (4°C). It was proposed that the separation efficiency of β-lactoglobulin could be optimized if: 1) aggregate size was maximized through pH manipulation (pH 5.2, 4°C) and 2) a support matrix that would accomodate complexes in excess of 340 nm was utilized.

Structure and functionality of β-lactoglobulin not only determines the separation kinetics for extraction protocals but also influences the physicochemical properties of the protein. Evaluation of kinetic parameters such as apparent dissociation constant (Kd) and protein/ligand molar binding ratio (n) for β-lactoglobulin variants A and B and retinoic acid was undertaken. In addition, the effect of digestive enzymes such as pepsin and trypsin on the bound complex was also investigated. Based on fluorometric quenching experiments β-lactoglobulin was found to bind retinoic acid with a molar protein/ligand ratio of 1:1. The retinoic bound β-lactoglobulin was examined in terms of its ability to withstand peptic and tryptic digestion at 37^0 C. Although resistant to pepsin digestion, β-lactoglobulin was highly susceptible to digestion by trypsin (in excess of 90% was digested). When β-lactoglobulin was bound to retinoic acid there was a significant reduction

in its digestibility (less than 25% of the protein was broken down) indicating that hydrophobic molecules such as retinoic acid may serve to protect β-lactoglobulin from specific digestive processes

6.2 RECOMMENDATIONS FOR FUTURE WORK

In order to commercialize β-lactoglobulin extraction, the speed and efficiency of separation must be further optimized. Changes in the biospecific subunit exchange chromatography technique could be coupled with a mechanical process such as ultrafiltration to increase flux and hence recovery. The major hurdle encountered in this study was the inability of the column to handle large order aggregates. If the biospecifc attachment was conducted by combining the ligand bound matrix and whey ouside a column and separating the resultant matrix/β-lactoglobulin using a large molecular weight cut-off membrane, the separation efficiency could be greatly increased. The matrix/protein could be dissociated using a percolation of low pH dissociating buffer. The parameters that were defined throughout this current body of work would aid in defining the conditions required for an alternative BSEAC apparatus.

In terms of the physicochemical properties of β-lactoglobulin further work is required to elucidate the binding limitations with hydrophobic molecules such as vitamin A. In addition, the application of β-lactoglobulin as a food vector transport protein requires experimentation that defines the stability of the protein/retinoic

complex under industrial processing conditions i.e. heat treatment, mechanical shear, etc.